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ORIGINAL PAPER

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Application of pentafluorophenyl hydrazine derivatives to the analysis of nabumetone and testosterone in human plasma by liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry

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Abstract Two carbonyl compounds, nabumetone and testosterone, were derivatized with pentafluorophenyl hydrazine (PFPH) and analyzed by atmospheric-pressure chemical-ionization mass spectrometry. The PFPH derivatives underwent dissociative electron capture in negative-ion APCI (ECAPCI) and gave intense $[M-20]^-$ ions in the mass spectra. In positive-ion APCI, the PFPH derivatives underwent efficient protonation and gave intense $[M+H]^+$ ions in the mass spectra. In CID, the major product ions of the $[M-20]^-$ ions in ECAPCI corresponded to the partial moiety of PFPH. In contrast, the major product ions of $[M+H]^+$ corresponded to the partial moiety of the analyte. By using selected reaction monitoring (SRM) detection, low pg of nabumetone (1 pg) and testosterone (7 pg) could be detected in both ECAPCI and positive-ion APCI. In comparison with the detection limits (SRM) of the underivatized analytes, use of the PFPH derivatives resulted in 2500-fold and 35-fold sensitivity enhancements for nabumetone and testosterone, respectively. The PFPH derivatives were applied to the analysis of nabumetone and testosterone in human plasma by both ECAPCI and positive-ion APCI and were found to enable detection of 0.1 ng mL^{-1} nabumetone in spiked plasma. For testosterone, endogenous testosterone in female plasma was detected in both ECAPCI and positive-ion APCI.

Keywords Pentafluophenylhydrazine · APCI · Electron capture · Protonation · Nabumetone · Testosterone

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Introduction

Electrospray ionization (ESI) is the most popular atmospheric-pressure ionization technique in LC–MS. However, it has been reported that the biological matrix present in these samples often results in analysis failures when ESI-MS is applied [1–4]. The analysis failure demonstrates the need to have a more selective sample-pretreatment procedure, better chromatographic separation, and deuterated internal standards in order to achieve accurate quantification.

Atmospheric-pressure chemical ionization (APCI) is another powerful atmospheric-pressure ionization (API) technique. In comparison with ESI, analyte signals in APCI are relatively insensitive to suppression by contaminants from the biological matrix [2, 4]. In APCI the flowing liquid is vaporized by a heated nebulizer and then ionized by passage through a zone in which an electrical discharge occurs. Usually one relies on the ions generated from the solvent to ionize the sample by protonation or deprotonation [5–8]. In addition to the proton-transfer reagent ions, it has been reported by Blair et al. [9, 10] that the corona discharge under negative-ion APCI conditions is also a source of gas-phase thermal electrons. Production of the thermal electron was thought to occur by displacement of electrons from the nitrogen sheath gas. The technique was referred as ''electron-capture atmospheric-pressure chemical ionization (ECAPCI)'', and was reported to provide sensitivity two orders of magnitude better than that of conventional APCI.

Because of the lack of a functional group with high gas-phase basicity or gas-phase acidity, carbonyl compounds, such as neutral steroids, generally do not provide the required sensitivity in APCI–MS. In positive-ion APCI introduction of a high proton-affinity group to the analyte is very effective in increasing sensitivity. The methyloxime derivative has been applied to the determination of neurosteroids in rat brain [11]. The

2,4-dinitrophenylhydrazine (DNPH) and *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) derivatives have been applied to the analysis of aldehydes and ketones in ambient air and tobacco smoke [12, 13]. The DNPH-derivatives have also been applied to the analysis of aldehydes and ketones in negative-ion APCI via deprotonation at the slightly acidic α-hydrogen atom of the hydrazine group [14, 15]. Recently, the 2-nitro-4-trifluoromethylphenyl hydrazine (2-NFPH) derivatives of oxosteroids were reported by Higashi et al. [16] for enhancement of the sensitivity of ECAPCI. Low pg detection limits were obtained. These derivatives have been applied to the analysis of oxosteroids in human plasma and rat brain by LC–ECAPCI–MS [16, 17].

Pentafluorophenylhydrazine (PFPH) is a commercially available reagent which has been applied as a derivatization agent for various carbonyl compounds in GC–ECD, GC–EI-MS and GC–ECNCI-MS analysis [18, 19]. Because the PFPH derivatives result in a high response in ECNCI-MS, very high sensitivity in ECAPCI is expected. Furthermore, because the PFPH derivatives also result in high gas-phase basicity, sensitivity increase in positive-ion APCI is also expected. In this work, the analytical potential of PFPH derivatives for analysis of neutral carbonyl compounds in both negative and positive-ion APCI was evaluated. Two carbonyl drugs, nabumetone and testosterone, were selected as the test compounds. The potential and limitation of the PFPH derivatives/APCI in the analysis of nabumetone and testosterone in human plasma are described.

Experimental

Materials

Solvents were all HPLC grade and were used without further purification. Methanol and dichloromethane were purchased from Mallinckrodt Baker (Paris, Kentucky, USA; 40361). Acetonitrile and *n*-hexane were purchased from Merck (Darmstadt, Germany) and J.T. Baker (Phillipsburg, USA; >95% purity). Pentafluorophenylhydrazine was purchased from Sigma (St Louis, USA). Nabumetone (>97% purity) and testosterone (>98% purity) were obtained from Divis Laboratories (Hyderabad, India) and Sigma, respectively. The 1 mg mL^{-1} analyte stock solutions and the 10 mg mL^{-1} pentafluorophenylhydrazine solution were prepared in methanol. The plasma samples were obtained from Mithra Bioindustry (Taipei, Taiwan). Female plasma sample was used as the blank plasma.

Preparation of PFPH standards

Standard solution (1 mg mL^{-1}, 10 μL) was transferred into a 16×100 mm screw-cap culture tube (Corning, USA). Pentafluorophenylhydrazine solution (10 mg mL^{-1}, 100 μL) and 100 μL 0.05 mol L^{-1} $HCl_{(aq)}$ were added. The solution was stirred for 15 s and the reaction was performed at 70°C for 30 min. After reaction, 3 mL *n*-hexane and 3 mL 0.1 mol L^{-1} $HCl_{(aq)}$ were added into the reaction mixture. The solutions were stirred for 3 min and centrifuged at 2800 rpm for 3 min. After freezing at −70°C for 20 min, the *n*-hexane layer was transferred to another culture tube and evaporated to dryness under nitrogen at room temperature. $CH_3OH_{(aq)}$ (80% *v*/*v*, 2 mL) was added to dissolve the dried residue. The PFPH-labeled solution corresponded to 5 μg mL^{-1} of intact drug.

Sample preparation of spiked plasma

An aliquot (1 mL) of blank plasma was fortified with the standards. *n*-Hexane–dichloromethane mixed solvent (1:1, *v*/*v*, 3 mL) was then added and stirred for 5 min. The sample was centrifuged at 2800 rpm for 5 min and was frozen at −70°C for 20 min. The organic layer was transferred to a 16×100 mm screw-cap culture tube and evaporated to dryness under nitrogen at room temperature. A total of 100 μL 0.05 mol L^{-1} $HCl_{(aq)}$ and 100 μL PFPH solution (10 mg mL^{-1}) were added. The reaction was performed at 70°C for 30 min. After reaction, 3 mL *n*-hexane and 3 mL 0.1 mol L^{-1} $HCl_{(aq)}$ were added to the reaction mixture. The sample was stirred for 5 min, centrifuged at 2800 rpm for 5 min and frozen at −70°C for 20 min. The *n*–hexane layer was transferred to another culture tube and dried with nitrogen flow. The dried residue was reconstituted with 200 μL 80% (*v*/*v*) $methanol_{(aq)}$ and 20 μL of the solution was injected for LC–MS–MS analysis.

Liquid chromatography

Liquid chromatography was performed on a Waters 2790 separation module equipped with an autosampler and a vacuum degasser (Waters, Milford, MA, USA). For evaluation of the detection limits a Hypersil MOS-2 C_8 silica-based column (4.6×50 mm, 5 μm, Cheshire, UK) was used. In ECAPCI the mobile phase was 90% $ACN_{(aq)}$ (*v*/*v*) and the flow rate was set at 1 mL min^{-1}. For underivatized analytes and PFPH derivatives in positive-ion APCI, the mobile phase was 90% $ACN_{(aq)}$ (*v*/*v*) containing 1 mmol L^{-1} formic acid and the flow rate was set at 1 mL min^{-1}. In the analysis of nabumetone and testosterone in spiked plasma, a Phenomenex Luna C_{18} silica-based column (4.6×250 mm, 5 μm, Torrance, USA) was used. The mobile phases were 88% $ACN_{(aq)}$ (*v*/*v*) in ECAPCI and 90% $ACN_{(aq)}$ (*v*/*v*) containing 1 mmol L^{-1} formic acid in positive-ion APCI. The flow rate was set at 1 mL min^{-1}.

Mass spectrometry

A Micromass Quattro Ultima triple-stage quadrupole mass spectrometer equipped with a Z-spray API source

(Micromass, Manchester, UK) was used in this study. In ECAPCI the discharge current was optimized at 20 μA for PFPH derivatives. The probe position in ECAPCI was found to be critical; the best responses were observed when the probe outlet was pointed to the corona. In positive-ion APCI, the optimized discharge current was 2 μA for PFPH derivatives and 7 μA for the underivatized analytes. In positive-ion APCI; the best responses were observed when the probe outlet was adjusted close to the sample cone. In both ECAPCI and positive-ion APCI, the desolvation temperature and source temperature were set at 500 and 80°C, respectively. The flow rates of nebulization gas, desolvation gas and cone gas were set at maximum, 400 and 100 L h^{-1}, respectively. The source cone voltage was set at 50 V. Collision-induced dissociation (CID) was performed using argon as the collision gas at 2.5×10^{-3} mbar in the rf-only hexapole. The collision energies were optimized to give the highest product ion intensities.

Results and discussion

Analysis of the PFPH derivatives by LC–APCI–MS

In this study, pentafluorophenylhydrazine (PFPH) was used as the derivatization agent to react with the carbonyl group of the selected analytes, nabumetone and testosterone. The PFPH-derivatization of nabumetone and testosterone is shown in Fig. 1. The synthesized PFPH derivatives were analyzed by both positive and negative-ion APCI. The mass spectra of nabumetone-PFPH and testosterone-PFPH are shown in Fig. 2. In positive-ion APCI, the PFPH derivatives underwent efficient protonation. The predominant ions corresponded to the $[M+H]^+$ ions of nabumetone-PFPH (m/z 409, Fig. 2a) and testosterone-PFPH (m/z 469, Fig. 2b). In negative-ion APCI, the PFPH derivatives did not undergo conventional deprotonation. The predominant ions were not the $[M-H]^-$ ions, but the $[M-20]^-$ ion for nabumetone-PFPH (m/z 388, Fig. 2c) and testosterone-PFPH (m/z 448, Fig. 2d). It is believed the PFPH derivatives underwent dissociative electron-capture ionization in negative-ion APCI. The $[M-20]^-$ ion was a result of loss of the HF molecule from the radical anion $[M]^{\cdot-}$, i.e. the $[M-HF]^-$ ion. In the negative-ion APCI mass spectrum of testosterone-PFPH, low abundance deprotonated ion $[M-H]^-$ (m/z 467) and $[M+12]^-$ (m/z 480) ions were also observed. The exact

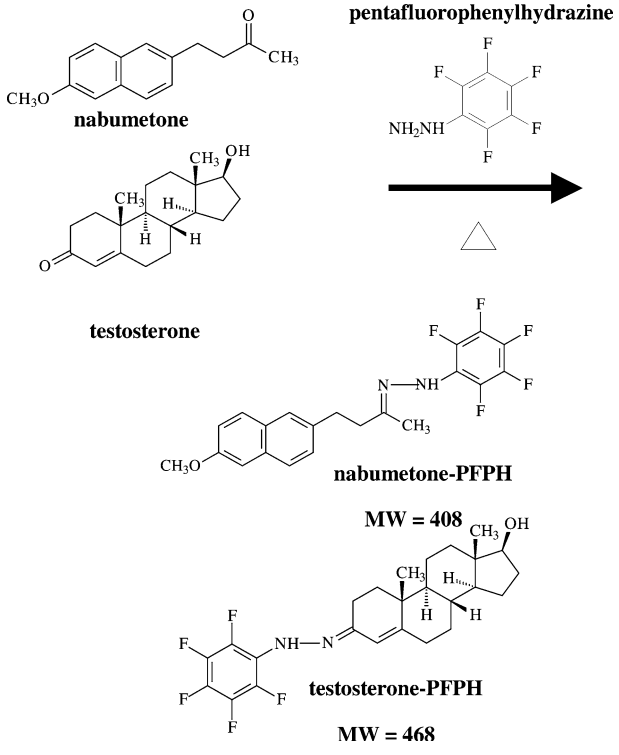

Fig. 1 Pentafluorophenylhydrazine derivatization of nabumetone and testosterone

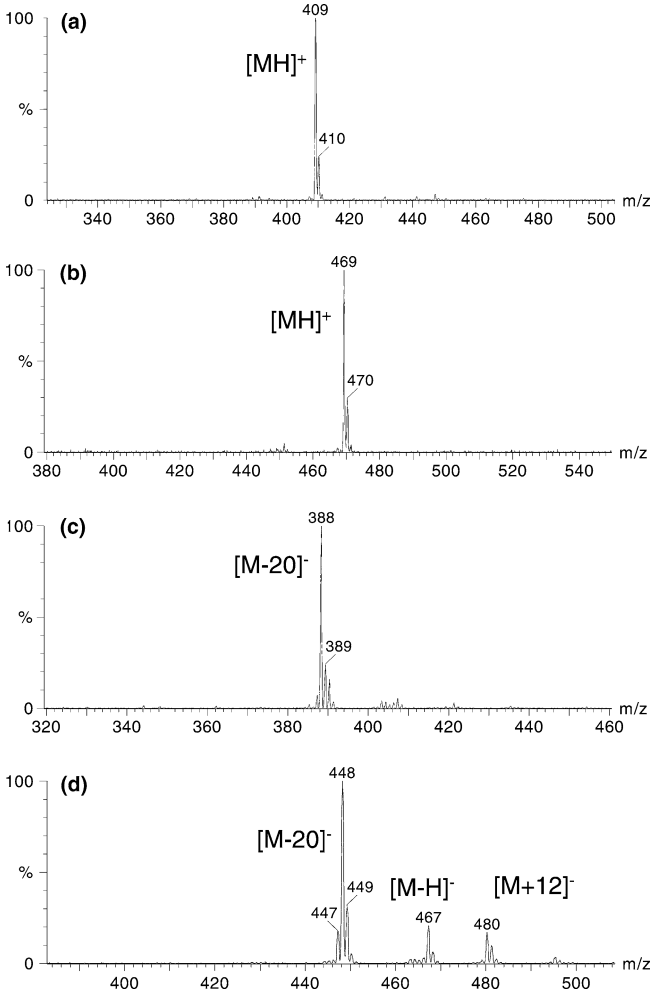

Fig. 2 Mass spectra of PFPH derivatives: (**a**) positive APCI mass spectrum of nabumetone-PFPH, (**b**) positive APCI mass spectrum of testosterone-PFPH, (**c**) negative APCI mass spectrum of nabumetone-PFPH, (**d**) negative APCI mass spectrum of testosterone-PFPH

identity of the $[M+12]^-$ ion is not known, but Higashi et al. have proposed it is the $[M-HF+O_2]^-$ ion [16].

The product ions of the PFPH derivatives

Collision-induced decomposition (CID) was performed to obtain the product-ion mass spectra of the $[M-20]^-$ and the $[M+H]^+$ ions of the PFPH derivatives. The product-ion mass spectra of the PFPH derivatives are shown in Fig. 3. In ECAPCI, the major product ions of nabumetone-PFPH (*m*/*z* 164) and testosterone-PFPH (*m*/*z* 149) corresponded to the partial moiety of the derivatization agent (Figs. 3a, b). The optimized collision energies were found to be 20 eV for nabumetone-PFPH and 40 eV for testosterone-PFPH. In positive-ion APCI, unlike the CID of the $[M-20]^-$ ion, analyte-specific product ions were obtained. The major product ions from the $[M+H]^+$ ion of nabumetone-PFPH and testosterone-PFPH included the partial moiety of the analytes (Figs. 3c, d). The optimized collision energies were found to be 25 eV for nabumetone-PFPH and 35 eV for testosterone-PFPH.

Detection limits of PFPH derivatives

The detection limits of PFPH derivatives and underivatized analytes in APCI were examined by selected reaction monitoring (SRM). The detection limits of nabumetone-PFPH and testosterone-PFPH in ECAPCI were established to be about 1 pg for nabumetone (Fig. 4a) and about 7 pg for testosterone (Fig. 4b). The detection limits achieved for the PFPH derivatives in ECAPCI are comparable with those obtained for the 2NFPH-derivatives (1–6 pg) [16, 17]. In positive-ion APCI the detection limits were very similar to results from ECAPCI (Figs. 4c, d). For the purpose of comparison, the detection limits of the underivatized analytes in positive-ion APCI were also measured. The detection limits were found to be about 2.5 ng for nabumetone and about 250 pg for testosterone (data not shown). These results are listed in Table 1. As can be seen, in both ECAPCI and positive-ion APCI, detection limits for the PFPH derivatives were about 2,500-fold (nabumetone) and 35-fold (testosterone) better than for the underivatized analytes in positive-ion APCI. It was noticed that sensitivity enhancement for

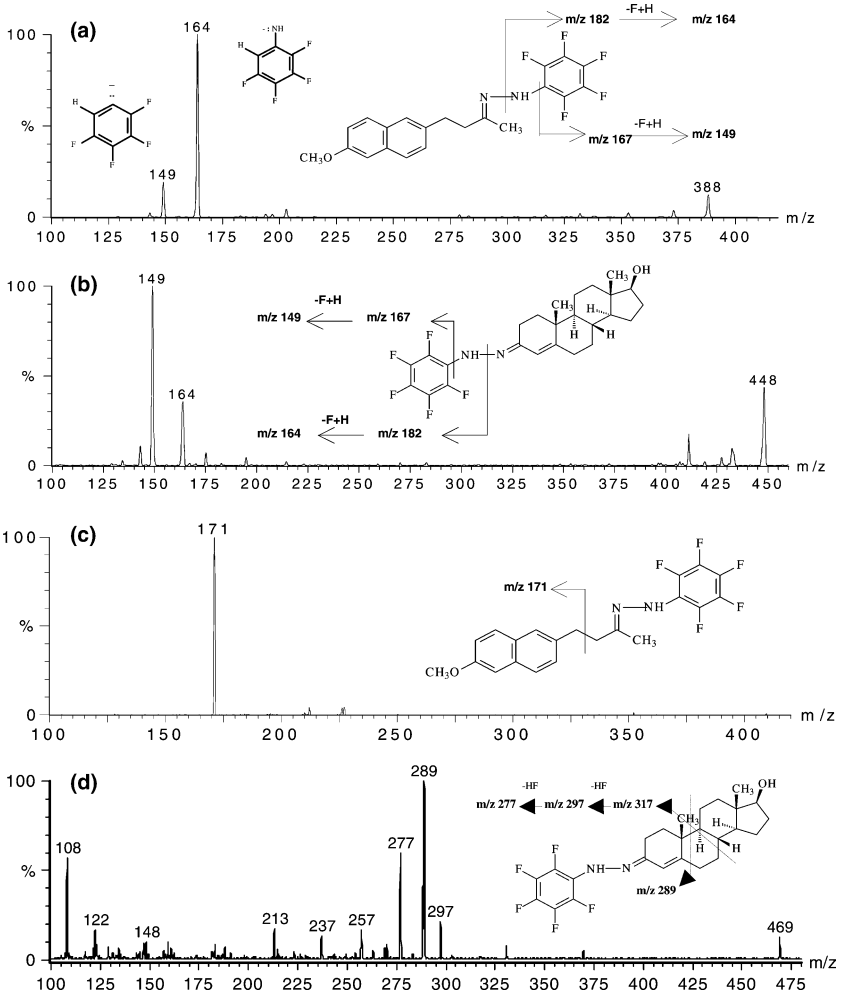

Fig. 3 Product ion spectra of PFPH derivatives: (**a**) nabumetone-PFPH in ECAPCI, (**b**) testosterone-PFPH in ECAPCI, (**c**) nabumetone-PFPH in positive-ion APCI, (**d**) testosterone-PFPH in positive-ion APCI

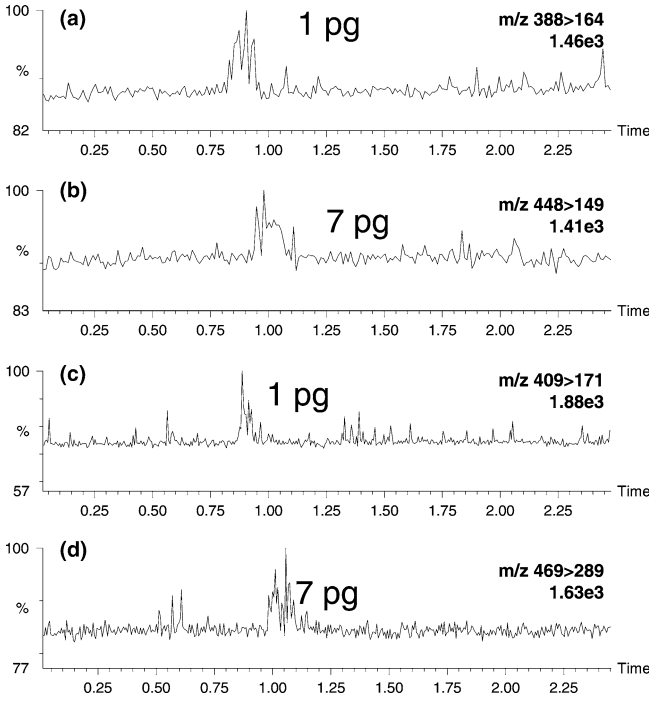

Fig. 4 Detection limits ($S/N \sim 3$) of PFPH derivatives in APCI: (**a**) 1 pg nabumetone in ECAPCI, (**b**) 7 pg testosterone in ECAPCI, (**c**) 1 pg nabumetone in positive-ion APCI, (**d**) 7 pg testosterone in positive-ion APCI

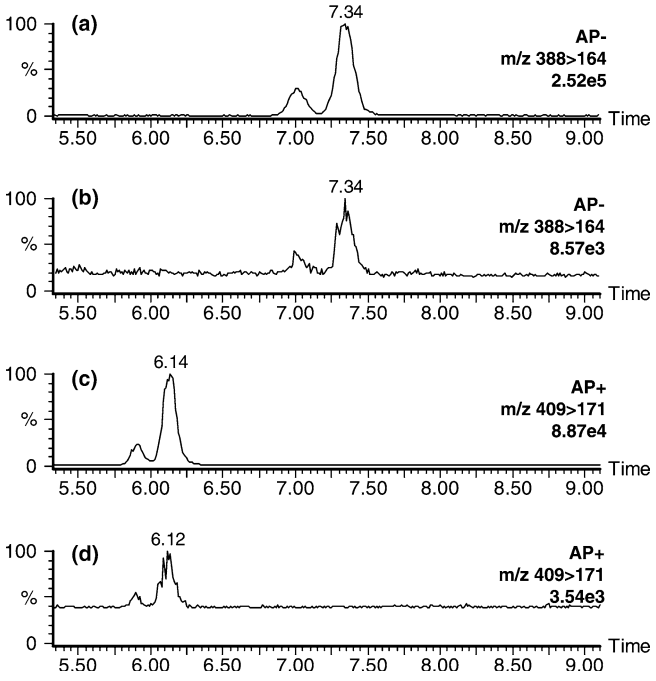

Fig. 5 LC–APCI–MS–MS mass chromatograms of nabumetone in spiked plasma samples: (**a**) 25 ng mL^{-1} spiked plasma in ECAPCI, (**b**) 0.1 ng mL^{-1} spiked plasma in ECAPCI, (**c**) 25 ng mL^{-1} spiked plasma in positive-ion APCI and (**d**) 0.1 ng mL^{-1} spiked plasma in positive-ion APCI

testosterone-PFPH was less than for nabumetone-PFPH. The conjugated carbonyl group (with higher proton affinity) is probably responsible for the higher response of the intact testosterone in positive-ion APCI.

Analysis of nabumetone and testosterone in plasma

To evaluate the analytical potential of the PFPH derivatives in APCI, the LC–MS–MS method was applied to the analysis of nabumetone and testosterone in human plasma. Nabumetone is a nonsteroidal anti-inflammatory drug for the symptomatic treatment of rheumatic and inflammatory conditions. After administration, nabumetone undergoes rapid and extensive metabolism. Because of the lack of a sensitive assay method, little pharmacokinetic information is available for intact nabumetone in biological fluids [20, 21]. Testosterone is one of the most important endogenous steroids and also the most commonly measured [16, 22–25]. Serum testosterone has been used to diagnose and monitor various disorders.

In the analysis of the selected compounds in plasma by ECAPCI, significant interferences were observed. The interference could be attributed to the fact that the product ions of PFPH derivatives in ECAPCI were not specific to the analytes. Thus, a longer LC column (25 cm) was used to reduce the interferences. In the analysis of nabumetone in spiked plasma, the mass transition of m/z 388 > 164 ($CV = 50$, $CE = 20$) was used for the detection of nabumetone-PFPH in ECAPCI. The mass chromatograms of 25 ng mL^{-1} nabumetone in spiked plasma are shown in Fig. 5a. The PFPH-hydrazones produced two geometrical isomers, i.e. E and Z isomers which were eluted at 7.0 and 7.3 min. As can be seen in Fig. 5b, the sensitivity of this technique was

Table 1 Detection limits ($S/N \sim 3$) of underivatized compounds and PFPH derivatives

	MW	Ionization mode	Mass transition (m/z)	Detection limits
Nabumetone[a]	228	Positive APCI	229 > 171	11.0 pmol (2.5 ng)
Nabumetone-PFPH[a]	408	Positive APCI	409 > 171	4.4 fmol (1.0 pg of nabumetone)
Nabumetone-PFPH[b]	408	ECAPCI	388 > 164	4.4 fmol (1.0 pg of nabumetone)
Testosterone[a]	288	Positive APCI	289 > 109	868.1 fmol (250.0 pg)
Testosterone-PFPH[a]	468	Positive APCI	469 > 289	24.3 fmol (7.0 pg of testosterone)
Testosterone-PFPH[b]	468	ECAPCI	448 > 149	24.3 fmol (7 .0 pg of testosterone)

[a]The mobile phase was 90% $CH_3CN_{(aq)}$ (v/v) containing 1 mmol L^{-1} formic acid
[b]The mobile phase was 90% $CH_3CN_{(aq)}$ (v/v)

capable of detecting 0.1 ng mL^{-1} nabumetone in spiked plasma.

Nabumetone in plasma was also analyzed by positive-ion APCI with the mass transition m/z 409 > 171 ($CV = 50$, $CE = 25$). The mass chromatogram of 25 ng mL^{-1} nabumetone in spiked plasma is shown in Fig. 5c. The geometrical isomers of nabumetone-PFPH were resolved at 5.9 and 6.1 min. Similar sensitivity to ECAPCI was obtained; 0.1 ng mL^{-1} of nabumetone in spiked plasma was also clearly detected (Fig. 5d).

The calibration curves for nabumetone were constructed by plotting the peak areas of the two isomers against the spiked concentration in plasma. Good linearity was obtained up to 25 ng mL^{-1} in both ECAPCI ($r^2 = 0.993$, Fig. 7a) and positive-ion APCI ($r^2 = 0.995$, Fig. 7b)

In the analysis of testosterone in plasma, the mass transition of m/z 448 > 149 ($CV = 50$, $CE = 40$) was used for the testosterone-PFPH detection in ECAPCI. The mass chromatogram of 25 ng mL^{-1} testosterone in spiked plasma is shown in Fig. 6a. The major isomer was observed at 9.0 min with a minor isomer at 8.3 min. However, it was observed that in the analysis of endogenous testosterone in the blank plasma, the peak

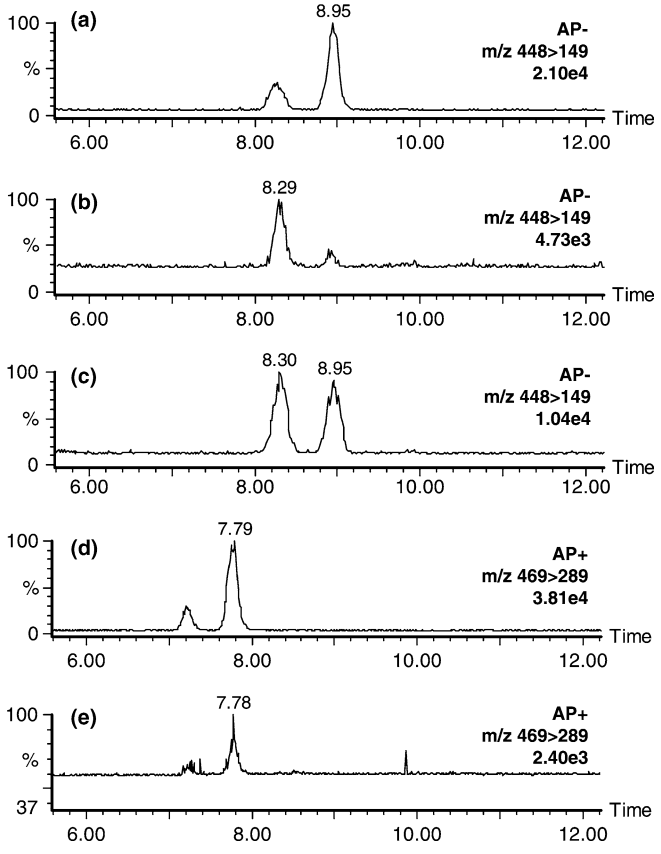

Fig. 6 LC–APCI–MS–MS mass chromatograms of testosterone in spiked plasma samples: (**a**) 25 ng mL^{-1} spiked plasma in ECAPCI, (**b**) female blank plasma in ECAPCI (**c**) male plasma in ECAPCI, (**d**) 25 ng mL^{-1} spiked plasma in positive-ion APCI, and (**e**) female blank plasma in positive-ion APCI

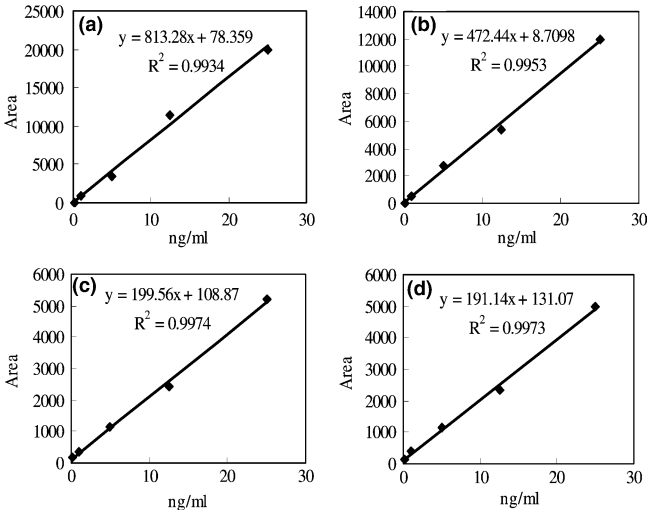

Fig. 7 Calibration plots for nabumetone and testosterone in spiked plasma: (**a**) nabumetone in ECAPCI, (**b**) nabumetone in positive-ion APCI, (**c**) testosterone in ECAPCI and (**d**) testosterone in positive-ion APCI. The analyte concentrations were spiked at 0.1, 1.0, 5.0, 12.5 and 25.0 ng mL^{-1}

at 8.3 min was significantly larger than the peak at 9.0 min (Fig. 6b). Therefore, it was speculated that interference in the plasma coeluted with the 8.3 min isomer. Two endogenous steroids with similar structure and identical nominal mass to testosterone, epitestosterone and dehydroepiandosterone (DHEA), were considered as the potential interference. Because the concentration of DHEA in female serum has been reported to be significantly higher than that of epitestosterone [26, 27], DHEA is considered as the more likely interference. The PFPH derivative of DHEA was synthesized and analyzed by LC–ECAPCI–MS. The major ions were the $[M-20]^-$ ion (m/z 448, base peak) and the $[M+12]^-$ ion (m/z 480, ~40%). In CID, the major product ion of the $[M-20]^-$ ion has the same m/z value (m/z 149) as the major product ion of testosterone-PFPH. In LC–MS–MS analysis, the DHEA-PFPH was detected with mass transition of m/z 448 > 149 ($CV = 50$, $CE = 40$) and eluted as a single peak at 8.3 min (data not shown). The observation of single isomer instead of two is more probably because of the much larger steric hindrance of the Z isomer of DHEA-PFPH in comparison with the E isomer. Based on this information, the interference at 8.3 min very probably arose from endogenous DHEA in the blank plasma. The small peak at 9.0 min in the mass chromatogram of the female blank plasma was endogenous testosterone (Fig. 6b). Its concentration was calculated to be about 0.73 ng mL^{-1}. For the purpose of comparison, a male plasma sample was also analyzed. The mass chromatogram of the male plasma sample is shown in Fig. 6c). As expected, a significantly higher concentration (~11.82 ng mL^{-1}) of endogenous testosterone was observed in the male plasma sample. The concentration of endogenous testosterone in human serum has been reported in the range of 0.17–2.07 ng mL^{-1} for female serum and

2.36–16.70 ng mL^{-1} for male serum [28]. The observed concentrations of endogenous testosterone in the plasma samples were in the normal range of endogenous testosterone in human serum.

Testosterone in plasma was also analyzed by positive-ion APCI. The testosterone-PFPH in positive-ion APCI was detected by using the mass transition of m/z 469 > 289 ($CV = 50$, $CE = 35$). The mass chromatogram of 25 ng mL^{-1} testosterone in spiked plasma is shown in Fig. 6d). The isomers of testosterone-PFPH were eluted at 7.2 and 7.8 min. The endogenous testosterone in the female blank plasma was also detected (Fig. 6e). Because the $[M+H]^+$ of DHEA-PFPH did not produce a product ion at m/z 289, the endogenous DHEA was not observed in the SRM trace of the blank plasma sample.

The calibration curves of testosterone were constructed by plotting the peak area of the major isomer (the peak at 9.0 min in ECAPCI and the peak at 7.8 min in positive-ion APCI) against the spiked concentration of testosterone in plasma. Good linearity was obtained up to 25 ng mL^{-1} in both ECAPCI ($r^2 = 0.997$, Fig. 7c) and positive-ion APCI ($r^2 = 0.997$, Fig. 7d).

Conclusions

Pentafluorophenylhydrazine derivatives have been used to enhance the sensitivity of analysis of nabumetone and testosterone by APCI–MS. The PFPH derivatives underwent efficient dissociative electron-capture ionization in negative-ion APCI and protonation in positive-ion APCI. The PFPH derivatives in ECAPCI and in positive-ion APCI were shown to result in comparable sensitivity. Low-pg detection limits were achieved by SRM detection in both ion modes. It was shown that the PFPH derivatives/APCI techniques were sensitive enough for detection of nabumetone and testosterone in human plasma at sub ng mL^{-1} levels.

One drawback of PFPH derivatives in ECAPCI is that the $[M-20]^-$ ions did not produce compound-specific product ions. Therefore, better separation or sample pretreatment might be required for PFPH derivatives in ECAPCI. Another drawback of PFPH derivatives is that two isomers could be generated for each analyte. Considering the better specificity of the product ion and similar sensitivity to ECAPCI, positive-ion APCI was considered a better choice than ECAPCI for analysis of PFPH derivatives. However, because the interference occurring in positive-ion APCI is not always the same as in ECAPCI, the ECAPCI can be a good alternative if there is significant interference in positive-ion APCI.

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