Journal of Chromatography, 593 (1992) 29–36
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2460

High-performance liquid chromatographic determination of monohydroxy compounds by a combination of pre-column derivatization and post-column reaction detection

Venkata K. Boppana*, Richard C. Simpson, Kathleen Anderson, Cynthia Miller-Stein, Timothy J. A. Blake, Bruce Y.-H. Hwang and Gerald R. Rhodes

Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, Mail Code L-712, King of Prussia, PA 19406 (USA)

ABSTRACT

A novel high-performance liquid chromatographic method was developed for the determination of monohydroxy compounds using the combined approach of pre-column derivatization and post-column reaction with fluorescence detection. Monohydroxy-containing drugs were modified by pre-column derivatization with propyl isocyanate (in pyridine, 50°C, 1 h) to form the corresponding *n*-propyl carbamate esters. Excess of reagent, reagent impurities and solvent were then removed by evaporation. Following chromatographic separation on a reversed-phase octadecylsilica column, the *n*-propyl carbamate ester derivative was subjected to post-column reaction and detection using alkaline hydrolysis to generate free propylamine, which was subsequently derivatized in-line using *o*-phthalaldehyde and 3-mercaptopropionic acid to form a fluorescent isoindole. The fluorescence emission of the isoindole product was measured at 455 nm following excitation at 340 nm. As propyl isocyanate is highly volatile and physico-chemically different to many drug molecules, problems associated with reagent impurities and reaction by-products were substantially minimized. The method was highly sensitive, allowing detection of sub-nanogram amounts of monohydroxy-containing drugs, hydroxy steroids and hydroxamic acids. The utility of this derivatization scheme was demonstrated by the highly sensitive measurement in human plasma of oxiracetam (4-hydroxy-2-oxo-1-pyrrolidineacetamide), an investigational drug intended for use in dementia and other memory disorders.

INTRODUCTION

The chemical modification of hydroxyl groups to improve the detectability of certain analyte molecules for high-performance liquid chromatographic (HPLC) applications typically involves pre-column derivatization with relatively large non-volatile chromophoric [1] or fluorescent reagents [2–7]. However, the use of such an approach to develop highly sensitive methodology for the determination of hydroxy-containing drugs at trace levels in biological fluids is complicated by several difficulties. The large excess of reagent concentration necessary to ensure complete and reproducible reaction with the analyte and the physico-chemical similarity between many chromophoric and fluorescent reagents and drug molecules necessitate extensive sample clean-up following reaction to remove excess of reagent and reagent impurities, in addition to derivatized interferences resulting from endogenous compounds. In most instances, clean-up approaches following derivatization are only partly successful, and interferences from reagent impurities limit the ultimate sensitivity that can be achieved.

In our approach, hydroxy-containing drugs were modified by pre-column derivatization with propyl isocyanate to form the corresponding *n*-propyl carbamate esters. Excess of reagent, reagent impurities and solvent were then removed by evaporation. Following chromatographic separation on a reversed-phase column, the *n*-propyl carbamate ester derivatives were subjected to post-column alkaline hydrolysis to generate free propylamine, which was subsequently derivatized in-line using *o*-phthalaldehyde and 3-mercaptopropionic acid to form a fluorescent isoindole. As propyl isocyanate is highly

0021-9673/92/$05.00

volatile and physico-chemically different to many drug molecules, problems associated with reagent impurities and reaction by-products were substantially minimized. The utility of this derivatization scheme was demonstrated by the sensitive measurement in human plasma of an investigational drug, oxiracetam (4-hydroxy-2-oxo-1-pyrrolidincacetamide), intended for use in dementia and other memory disorders.

EXPERIMENTAL

Materials

Oxiracetam (SK&F 107823, I, Fig. 1) and the internal standard (ISF 2839, I.S.) were obtained from ISF Laboratories (Milan, Italy). HPLC-grade water (Milli-Q water purification system; Millipore, Bedford, MA, USA) was used in the preparation of standard solutions, buffers and mobile phase. Analytical-reagent grade glacial acetic acid and sodium hydroxide were purchased from Mallinckrodt (Paris, KY, USA), HPLC-grade methanol and acetonitrile from J. T. Baker (Phillipsburg, NJ, USA), silylation-grade pyridine and *o*-phthalaldehyde (OPA) from Pierce (Rockford, IL, USA), propyl isocyanate (99%), phenyl isocyanate and benzohydroxamic acid from Aldrich (Milwaukee, WI, USA) and 3-mercaptopropionic acid and all steroid compounds from Sigma (St. Louis, MO, USA). All other chemicals were analytical-reagent grade. Phenylboronic acid (PBA) solid-phase extraction columns (100 mg/ml) were purchased from Analytichem International (Harbor City, CA, USA).

o-Phthalaldehyde reagent solution

Sodium hydroxide (2 g) was first dissolved in 1 l of degassed HPLC-grade water and then 2 ml of freshly prepared methanolic solution of *o*-phthalaldehyde (2 mg/ml) and 80 μl of 3-mercaptopropionic acid were added. The solution was filtered through a 0.45-μm nylon 66 filter. The reagent was stable for 48 h at room temperature.

Fig. 1. Structures of oxiracetam (I) and the internal standard (I.S.).

Standard solutions

The stock (1 mg/ml) and working standard solutions (100, 10, 1 and 0.1 μg/ml) of oxiracetam and the internal standard were prepared in water. The solutions were stable for 1 month. The stock and standard solutions of steroids and hydroxamic acids were prepared in pyridine.

Pre-column derivatization of monohydroxy compounds

To the tubes containing oxiracetam or monohydroxy-containing analytes, anhydrous pyridine (200 μl) and propyl isocyanate (50 μl) were added and the solution was vortex mixed. The tube was sealed with Parafilm and placed in a water-bath maintained at 50°C for 1 h. The sovlents were evaporated at 50°C under nitrogen and the residue was dissolved in the mobile phase.

Mass spectrometric (MS) analysis of n-propyl carbamate derivatives

LC–MS was performed using an HP 1090A HPLC system (Hewlett-Packard, Waldbronn, Germany) interfaced to a Finnigan MAT (San Jose, CA, USA) TSQ 70 triple quadrupole mass spectrometer via a Finnigan MAT thermospray ionization (TSP) interface. Chromatographic separations were carried out on a Hypersil ODS column (10 cm × 4.6 mm I.D.) (Hewlett-Packard) using a mobile phase of ammonium formate (0.1 *M*, pH 3.8)–acetonitrile at a flow-rate of 1 ml/min. Subsequent to injection (3.5 min), the concentration of acetonitrile was increased linearly from 0 to 35% over a period of 25 min, held at that level for 2 min and then cycled back to the initial conditions. Mobile phase components were filtered through a 0.2-μm nylon 66 filter and degassed before use. The TSP vaporizer temperature was 108°C and the jet temperature was 260°C.

LC–MS with continuous-flow fast atom bombardment (CF-FAB) ionization was performed using the same HPLC system as above interfaced to the Finnigan MAT TSQ 70 via a Finnigan MAT BIO Probe ion source. Ionization was accomplished using xenon as the bombarding gas and a VCR Group (San Francisco, CA, USA) saddle-field gun

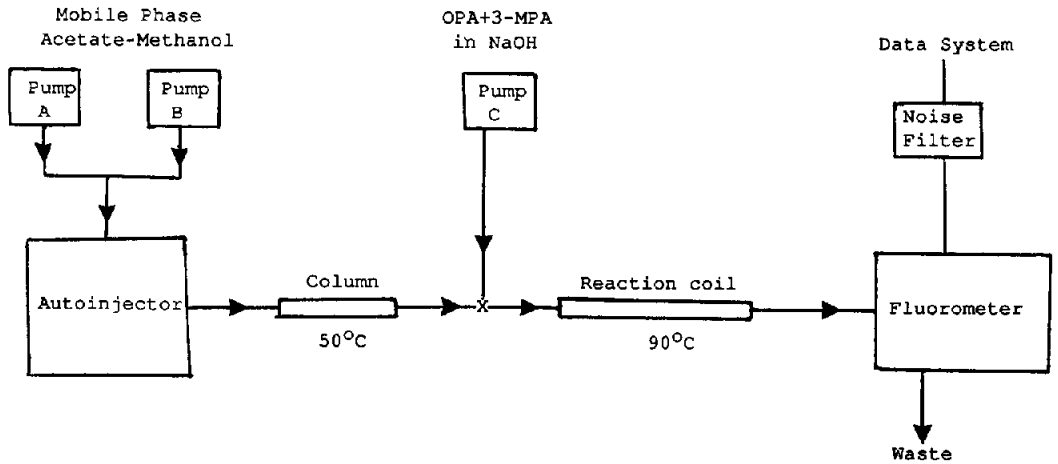

Fig. 2. Schematic diagram of the chromatographic system.

operated at 6 kB and 2 mA. Isocratic chromatographic separations were carried out on Zorbax Rx octyl column (15 cm × 2.1 mm I.D.) (Mac Mod Analytical, Chadds Ford, PA, USA) using a mobile phase consisting of 60% water and 40% organic modifier [acetonitrile–methanol (50:7, v/v)]. The mobile phase contained 5% glycerol and 0.1% trifluoroacetic acid. The flow-rate of the mobile phase was 250 μl/min. A flow splitter was placed before the UV flow cell, which reduced the flow of mobile phase into the mass spectrometer to 2 μl/min. The probe tip temperature was held at 26°C to prevent freezing. For all analyses, the mass spectrometer was operated in alternating positive–negative ion full-scan modes.

High-performance liquid chromatography

The HPLC system (Fig. 2) consisted of a Hitachi 665A-12 high-pressure gradient semi-micro solvent delivery system (EM Science), a post-column reactor module (PCRS Model 520; ABI Analytical, Ramsey, NJ, USA) and a Hitachi F-1000 fluorescence detector (EM Science). Chromatographic separations were carried out on a 25 cm × 2.0 mm I.D., octadecylsilica (5 μm) column (Ultrasphere) (Beckman Instruments, Palo Alto, CA, USA), maintained at 50°C, at a flow-rate of 300 μl/min. The initial mobile phase composition was 0.05 *M* acetate buffer (pH 6.0)–methanol (90:10, v/v). Following injection, the methanol concentration was held at 10% for 5 min, then raised to 20% over a period of 4 min, held for 1 min, then increased to 50% in 1 min, held at 50% for 5 min and cycled back to the initial conditions in 1 min. The system was equilibrated at the initial mobile phase composition for 14 min before injecting the next sample. Mobile phase components were filtered through a 0.2-μm nylon 66 filter and degassed before use. Samples were injected using an HPLC autosampler (WISP, Model 710B; Waters Assoc., Milford, MA, USA). The post-column reactor module contains two independently heated zones which are used, in this instance, as a column heating chamber and a reaction coil heating block. One additional pump (Model 114, Beckman Instruments) was utilized to deliver the OPA reagent solution at a flow-rate of 200 μl/min to the post-column reactor where they were mixed with the column effluent utilizing a low-dead-volume mixer. Following formation of the fluorescent reaction product, detection was accomplished utilizing excitation at 340 nm while monitoring the fluorescence emission at 455 nm. The chromatographic data were collected with a computerized automated laboratory system (Access*Chrom; PE-Nelson, Cupertino, CA, USA).

RESULTS AND DISCUSSION

Initial attempts to derivatize monohydroxy-containing drugs with various commercially available fluorogenic reagents, such as 9-anthroylnitrile [3], 7-methoxycoumarin-3-(and 4-)-carbonyl azides [4]

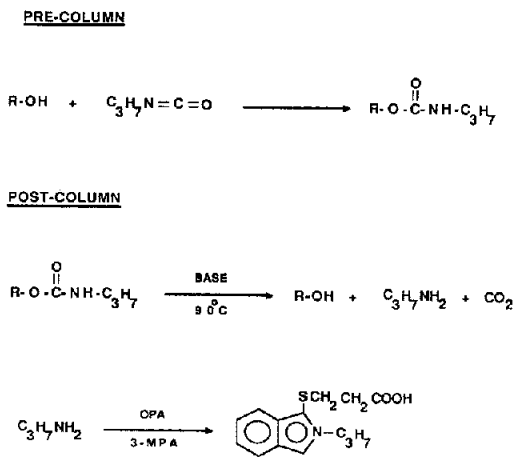

Fig. 3. Schematic representation of reactions showing the pre-column conversion of hydroxy compounds to *n*-propyl carbamate compounds and post-column conversion of the latter into fluorescent isoindole structures.

and 7-[(chlorocarbonyl)methoxyl]-4-methylcoumarin [6], were problematic owing to the difficulties associated with the stability of these reagents. Various environmental factors, such as moisture, temperature and light, affect the stability during storage and lead to very short shelf lives for these reagents. These problems often require either custom synthesis or repurification of reagents. Moreover, the large excess of reagent concentration employed to ensure the complete and reproducible reaction with the analyte and the physico-chemical similarity between the derivatized products and the reagent typically necessitate extensive sample purification following reaction to remove excess of reagent and numerous reagent impurities, in addition to derivatized interferences due to endogenous compounds. In our approach, hydroxy-containing drugs were first modified by pre-column derivatization with propyl isocyanate to form the corresponding *n*-propyl carbamate esters. Following removal of the excess or reagent and solvent by evaporation, the carbamate esters were separated by reversed-phase HPLC and then subjected to simultaneous post-column base hydrolysis and derivatization of the liberated propylamine with OPA–thiol (Fig. 3).

Several aliphatic and aromatic isocyanates were examined for pre-column derivatization of monohydroxy compounds to form carbamate esters. Based on the use of oxiracetam as a model compound, propyl isocyanate was chosen owing to its superior physico-chemical properties, such as ease of handling, safety, reactivity and ease of removal from the reaction medium. Propyl isocyanate is a liquid which is less volatile than its lower homologues, and can be handled safely. In comparison with phenyl isocyanate, however, the volatility and ease of hydrolysis of carbamate esters favored propyl isocyanate for pre-column derivatization of monohydroxy compounds.

The formation of the *n*-propyl carbamate ester of oxiracetam was confirmed by both post-column reaction detection via fluorescence response and mass spectral evidence. The *n*-propyl carbamate ester of oxiracetam was subjected to LC-TSP-MS analysis, and the positive ion mass spectrum is shown in Fig. 4A. The protonated molecular ion ($[M+H]^+$, m/z = 244) was observed as the base peak of the spectrum. The corresponding ammonium, sodium and potassium adduct ions were observed at m/z 261, 266 and 282, respectively. Loss of propyl isocyanate ($C_3H_7N=C=O$) from the $[M+H]^+$ ion yielded the ion at m/z 159. The ion at m/z 141 indicated loss of the elements of *n*-propyl carbamate. The negative ion mass spectrum is shown in fig. 4B. It shows a base peak ion at m/z 288, which corresponds to the formate molecular anion adduct ($[M+HCOO]^-$) of the *n*-propyl carbamate derivative of oxiracetam. Loss of *n*-propyl ketene from the ion at m/z 288 yielded the ion at m/z 203.

The pre-column formation of *n*-propyl carbamate esters of hydroxy compounds was optimized by using oxiracetam as a model compound. As oxiracetam was highly hydrophilic in nature, pyridine was chosen to conduct the reaction. The reaction was carried out at different temperatures for various times and the HPLC with post-column hydrolysis and fluorescence detecion was used to obtain peak heights. Derivatized oxiracetam was separated by gradient elution HPLC using the mobile phase conditions described under Experimental. By varing the pre-column reaction temperature between 30 and 80°C, it was observed that the reaction rate was optimum when the temperature of the reaction was maintained between 50 and 60°C. The effect of pre-column reaction time was also examined by allowing the reaction to proceed from 30 to 240 min at 50°C. The results from this experiment indicated

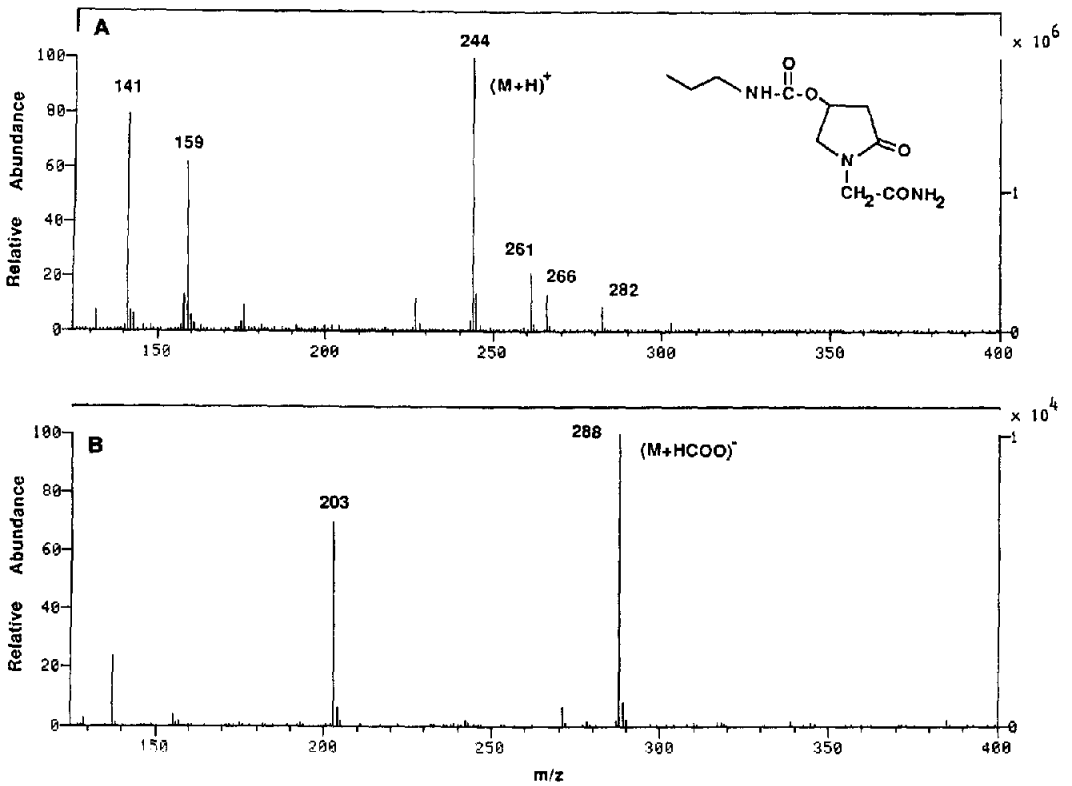

Fig. 4. LC–TSP-MS of oxiracetam *n*-propyl carbamate: (A) positive ion mass spectrum; (B) negative ion mass spectrum.

that a reaction time of 60 min was necessary to obtain optimum yields of the carbamate derivative. Using ^{3}H-labelled oxiracetam, and following the disappearance of oxiracetam and the formation of the carbamate ester in the reaction mixture by isocratic HPLC with radiometric detection (Beckman), it was determined that the conversion of oxiracetam to the *n*-propyl carbamate derivative was quantitative.

Post-column fluorescence detection of carbamates typically involves a two-stage (two-pump) reaction system [8]. The carbamate esters were first hydrolyzed to release a primary amine, which was subsequently derivatized on-line with *o*-phthalaldehyde and a thiol to form highly a fluorescent substituted isoindole. Of the several approaches available for post-column hydrolysis of carbamate esters, we selected base hydrolysis owing to its ease and simplicity. Moreover, it has been shown that *o*-phthalaldehyde and 3-mercaptopropionic acid can both be added to the hydrolytic reagent solution [9], eliminating the need for a second post-column pump. This allows for the simultaneous hydrolysis and fluorescence detection of carbamates without compromising the sensitivity and offers the same advantages as offered by solid-phase reactors [10,11] and photolytic hydrolysis methods [12].

In order to optimize the reaction conditions for the 2.0 mm I.D. reversed-phase columns used here, certain post-column reaction parameters were examined using the *n*-propyl carbamate ester of oxiracetam as a model substrate. In these experiments, the post-column flow-rate and concentration of OPA and thiol reagent solution were maintained as described under Experimental. The post-column reaction conditions for the concentration of sodium hydroxide, temperature and reaction time were then optimized by injecting 10 ng of derivatized oxiracetam on to the column and monitoring the intensity of the fluorescence signal obtained.

The effect of base concentration was examined by varying the sodium hydroxide concentration from 0.01 to 0.3 *M*. The results indicated that a base concentration of 0.05 *M* provided the optimum fluorescence signal for derivatized oxiracetam. The effect of temperature on the post-column reaction was then examined by varying the reaction coil temperature from 40 to 110°C. A reaction temperature of 90°C was found to be optimum for the post-column derivatization. The effect of reaction coil volume (0.5–2.0 ml) on the post-column reaction was also examined. The 1.0-ml reaction coil provided the optimum chromatographic peak height without a significant change in peak broadening. These conditions were utilized in subsequent work.

In order to establish the applicability of this approach for the routine determination of monohydroxy compounds, the linearity and precision of the combined pre- and post-column reaction detection system were examined. The linearity was evaluated by analysis of a series of standard solutions of oxiracetam, and a linear response over the range 2–2000 ng injected on-column was obtained. The curves were highly reproducible and correlation coefficients were typically >0.999. The precision of the method was determined by repetitive pre-column derivatization of a standard solution of oxiracetam followed by post-column reaction detection and measurement of the resulting chromatographic peak heights. The method displayed excellent precision, yielding relative standard deviations (R.S.D) of <3% ($n=6$). The limit of detection (signal-to-noise ratio = 3) for oxiracetam was 0.5 ng.

The application of the method to the determination of monohydroxy-containing compounds was exemplified by the HPLC assay developed to determine the concentration of oxiracetam in human plasma. The assay involved precipitation of plasma protein with acetonitrile followed by application of the supernatant to a phenylboronic acid column and collection and evaporation of the breakthrough liquid containing the analyte. Other endogenous polyhydroxy compound interferences remained on the column. The residue obtained was then subjected to the pre-column derivatization and post-column reaction detection scheme described above. Typical chromatograms from extracts of a drug-free plasma sample and a plasma sample spiked with 1 μg/ml of oxiracetam are shown in Fig. 5. The

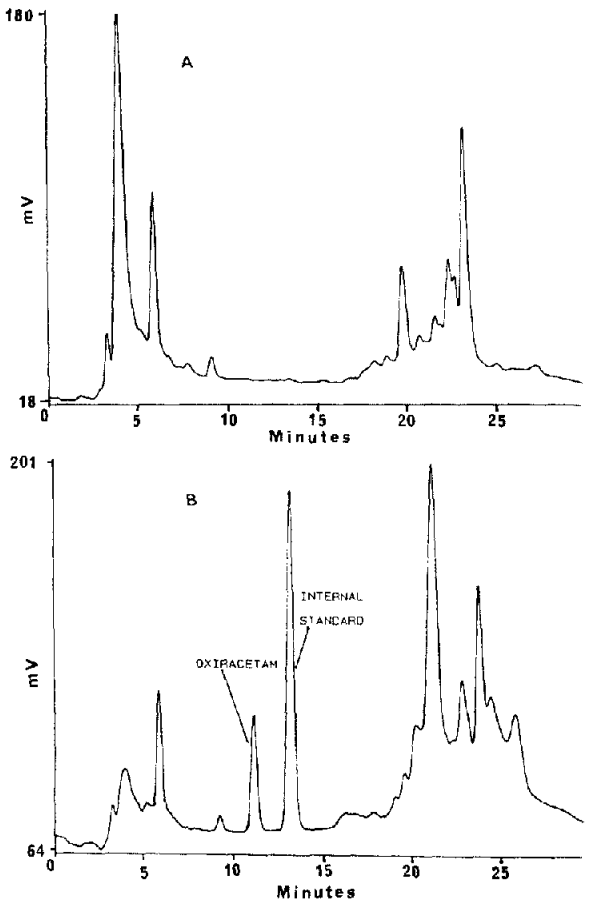

Fig. 5. Chromatograms of extracts of (A) drug-free plasma and (B) plasma sample spiked with 1 μg/ml of oxiracetam.

retention times for oxiracetam and the internal standard were 10.1 and 12 min, respectively. The chromatograms displayed no endogenous interfering peaks in the region of oxiracetam or the internal standard. Under the conditions utilized in this assay, the lowest concentration of oxiracetam that could be determined in 0.2 ml of plasma was 40 ng/ml, which corresponds to an injected amount of *ca.* 2 ng. The correlation coefficients for plasma calibration graphs were typically >0.99.

The method reported here may have general utility. This approach has also been applied to the HPLC detection of other classes of monohydroxy compounds such as steroids and hydroxamic acids. Of the various hydroxy steroids subjected to this carbamate derivatization procedure, aromatic ste-

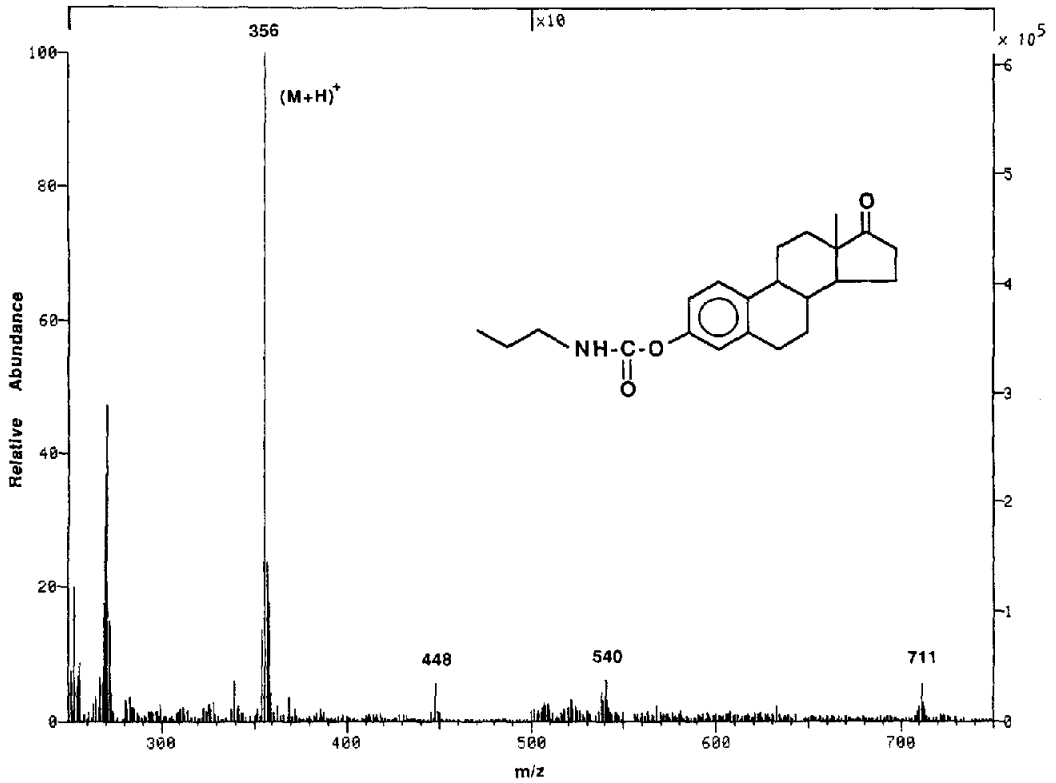

Fig. 6. LC–CF-FAB-MS of estrone *n*-propyl carbamate (positive ion mass spectrum).

roids, such as estrone, and steroids with a primary hydroxy group, such as cortisone, readily yielded the corresponding *n*-propyl carbamates, which were subsequently measured by HPLC with post-column reaction detection. The formation of estrone *n*-propyl carbamate was also confirmed by the positive ion CF-FAB mass spectrum (Fig. 6). The protonated molecular ion ($[M+H]^+$, m/z = 356) was observed as the base peak in the mass spectrum. The corresponding glycerol adduct ions were observed at m/z 448 and 540. A protonated dimer of the molecular ion ($[2M+H]^+$) was also observed at m/z 711. The ion at m/z 253 indicated loss of $C_3H_7{=}N{=}C{=}O$. Steroids such as progresterone, cholic acid and testosterone did not form the corresponding *n*-propyl carbamates under these conditions, presumably owing to steric hindrance effects [13]. Benzohydroxamic acid also readily yielded the *n*-propyl carbamate derivative, which was detectable by post-column reaction and fluorescence detection. The results of these studies suggest that certain hydroxy compounds with complex structures may require additional optimization of the reaction conditions used to form the corresponding *n*-propyl carbamate derivatives. To achieve this, one may have to use different solvents, adjust the basicity of the reaction medium or employ various catalysts. As with many pre-column derivatization approaches, the potential reactivity of other functional groups with *n*-propyl isocyanate may lead to the formation of other by-products, thus complicating the analysis. In this instance, reaction of primary and secondary amine groups with *n*-propyl isocyanate has been observed, leading to the formation of substituted urea products.

The combined pre- and post-column reaction detection system described here provided a highly sensitive method for the fluorescence detection of certain monohydroxy compounds such as oxiracetam. The sensitivity of the method allowed its successful application to the determination of oxiracetam in human plasma samples. In addition, the method

may have some general utility as highly sensitive detection was also achieved with certain steroid compounds and hydroxamic acids.

REFERENCES

1 F. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973)) 2310.
2 J. Goto, S. Komatsu, N. Goto and T. Nambara, *Chem. Pharm. Bull.*, 29 (1981) 899.
3 J. Goto, N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara, *Anal. Chim. Acta*, 147 (1983) 397.
4 A. Takadate, M. Iwai, H. Fujino, K. Tahara and S. Goya, *Yakugaku Zasshi*, 103 (1983) 962.
5 A. Takadate, M. Irikura, T. Suehiro, H. Fujino and S. Goya, *Chem. Parm. Bull.*, 33 (1985) 1164.
6 K. Karlsson, D. Wiesler, M. Alasandro and M. Novotny, *Anal. Chem.*, 57 (1985) 229.
7 Y. Tsuruta and K. Kohashi, *Anal. Chim. Acta*, 192 (1987) 309.
8 H. A. Moye, S. J. Scherer and P. A. St. John, *Anal. Lett.*, 10 (1977) 1049.
9 B. D. McGarvey, *J. Chromatogr.*, 481 (1989) 445.
10 L. Nondek, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 54 (1983) 141.
11 L. Nondek, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 282 (1983) 141.
12 C. J. Miles and H. A. Moye, *Anal. Chem.*, 60 (1988) 220.
13 J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, *J. Chromatogr.*, 276 (1983) 289.