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Determination of fenbufen and its metabolites in serum by reversed-phase high-performance liquid chromatography using solid-phase extraction and on-line post-column ultraviolet irradiation and fluorescence detection

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Abstract

An improved analytical method for the detection and quantification of fenbufen and its two major metabolites is described. The assay consists of reversed-phase high-performance liquid chromatography and post-column irradiation with ultraviolet light and fluorescence detection. A highly selective chromatography separation was established on a cyanopropyl column at ambient temperature with a flow-rate of 0.5 ml/min. The analytes of interest were isolated from serum using a Bond-Elut C_{18} column with high recovery and selectivity. The fluorescence response of all three analytes upon UV irradiation was investigated. The post-column UV irradiation was optimized and the effect of irradiation time on the fluorescence response was determined for all three analytes. The detection limits were 10 ng/ml for each analyte using 1 ml of serum. Linear calibration curves from 50 to 375 ng/ml for all three analytes show coefficients of determination of 0.99. Precision and accuracy of the method were within 3.9–6.5 and 5.1–7.4% for fenbufen, 3.5–6.4 and 4.9–6.3% for metabolite II (expressed as lactone III) and 5.4–7.4 and 2.6–7.4% for metabolite IV, respectively.

Keywords: Fenbufen; 3-(4-Biphenylhydroxymethyl)propionic acid; 4-Biphenylacetic acid

1. Introduction

Post-column photochemical reaction detection has been used frequently for converting non-responding or poorly responding analytes to species that can be detected with increased specificity and selectivity compared to conventional HPLC detection methods [1–16]. A number of reviews have been published on the subject [17–20]. The photochemical reactor is easy to construct and a commercial version is also available.

Fenbufen (I), an orally active non-steroidal anti-inflammatory agent, is commonly used for rheumatoid arthritis. Its two major serum metabolites 3-(4-biphenylhydroxymethyl)propionic acid (II) and 4-biphenylacetic acid (IV) have similar activity [21]. Methods previously reported in the literature include GC [22] and HPLC with UV detection [23,24]. The GC method is tedious and involves a back-extraction, derivatization and thin-layer chromatographic separation prior to injection. The HPLC methods are based on UV detection and report retention times up

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to 22 min using 45°C column temperature and 2 ml plasma sample, tedious liquid–liquid extraction procedure and limits of detection of 500 ng/ml.

This paper describes a reversed-phase HPLC method using post-column UV irradiation and solid-phase extraction (SPE) to measure low ng/ml concentrations of fenbufen and its metabolites in serum with good sensitivity, selectivity and fast chromatographic run time. The method is linear up to 375 ng/ml of fenbufen and its metabolites. The assay procedure possesses the required sensitivity to be useful for monitoring blood levels of fenbufen doses of 600 mg.

2. Experimental

2.1. Reagents

Fenbufen and ketoprofen (internal standard) were obtained from Sigma (St. Louis, MO, USA). The two metabolites II and IV were donated by Lederle Labs. (Pearl River, NY, USA). Drug-free serum was obtained from Fisher Scientific (Cat No. 3160-34, Orangeburg, NY, USA). The solvents used were all HPLC grade. Acetonitrile, absolute methanol and concentrated phosphoric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA). All chromatographic solutions were filtered through a 0.45-μm filter (Alltech Associates, Deerfield, IL, USA).

2.2. Preparation of stock and standard solutions

Metabolite II is referred to hereafter in this paper as lactone III unless otherwise specified. A stock solution of lactone III was prepared by the method described elsewhere [20]. It involved saturation of metabolite II in water at 40°C with an excess of HCl added. After 5 min, III was extracted into cyclohexane–diethyl ether (7:3, v/v). The organic layer was evaporated under nitrogen stream and the lactone crystals were collected. A fresh solution of III was prepared each day since the lactone was not stable in methanol.

Individual stock solutions (100 μg/ml) of fenbufen (I), lactone III, metabolite IV and the internal standard ketoprofen (V) (1 mg/ml) were prepared in absolute methanol. A working standard solution of 10 μg/ml was prepared from the individual stock solutions of the three analytes. Appropriate volumes of the three analytes (50–350 μl) and internal standard (6 μl) were pipetted into a 1-ml volumetric flask and drug free serum added to volume to give final serum concentrations of 50, 100, 150, 200, 250, 300 and 375 ng/ml of each analyte and 6 μg/ml of the internal standard.

3. Instrumentation

3.1. HPLC system

A Model 110B HPLC pump (Beckman, Fullerton, CA, USA) and a Model 728 autosampler (Micromeritics, Norcross, GA, USA) equipped with a 100-μl loop were used for the analysis. The stationary phase was a cyanopropyl column (Brownlee Spheri-5 cyano 5 μm, 100×4.6 mm I.D., Applied Biosystems, Foster City, CA, USA). The mobile phase consisted of water–absolute methanol–acetonitrile–phosphoric acid (56.5:22:21:0.5, v/v). The flow-rate was 0.5 ml/min and the column was at ambient temperature (22±1°C). A Model 1046A programmable fluorescence detector (Hewlett Packard, Avondale, PA, USA) was used with an excitation wavelength of 248 nm and an emission filter of 335 nm. A SP 4290 integrator (Spectra Physics, San Jose, CA, USA) was used to record each chromatogram and peak area responses.

3.2. Post-column photochemical reactor

The photochemical reactor was equipped with an SC3-9 UV lamp and SCT-4 power supply (UVP, San Gabriel, CA, USA). The irradiation coil (790 cm× 0.3 mm I.D.) was made from teflon (PTFE) tubing (Anspec, Ann Arbor, MI, USA). The reaction coil was knitted in a manner similar to that described in the literature, with minor modifications [25]. In contrast to the reported method, where the knitter used a 3- or 4-peg "Strickliesel" which resulted in a dense rope like configuration, the coil used in this experiment was knitted around 6 pegs which resulted in a hollow cylinder. The coil was snug onto the surface of the lamp in a sleeve like manner. A 3-in-diameter fan (Rotron, Woodstock, NY, USA)

was provided for air cooling. A box (31.5×16.5×15.5 cm) constructed from galvanized sheet steel and perforated masonite was used to house the UV lamp, irradiation coil and fan.

3.3. Assay procedure and preparation of standard calibration curve

A 1-ml volume of distilled water, 20 μl of saturated ammonium sulphate solution and 60 μl of concentrated HCl were added to 1-ml serum samples containing fenbufen and its metabolites and internal standard. For an unknown sample, 6 μl of the internal standard should be added to 1 ml of the serum sample followed by the addition of 1 ml of distilled water, 20 μl of saturated ammonium sulphate and 60 μl of HCl. The samples were vortex-mixed for 3 min and then passed through a 1-ml C_{18} Bond-Elut SPE column attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products, Harbor City, CA, USA) which was previously conditioned with 1 ml of methanol. The column was washed with 3×1 ml of water and allowed to dry for 3 min. The analytes of interest were eluted with 5×500 μl of methanol and evaporated under a nitrogen stream. The residue was dissolved in 1 ml of the mobile phase and a 100-μl aliquot was injected into the HPLC system. Linear calibration curves were constructed in the range of 50–375 ng/ml using 50, 100, 150, 200, 250, 300 and 375 ng/ml concentration of each analyte. Linear regression analysis of drug/internal standard peak-area ratios versus concentration gave slope and intercept data for each analyte which were used to calculate the concentration of each analyte in the serum samples.

For absolute recovery experiments of each analyte, spiked samples were compared to unextracted stock solutions. Drug peak-area ratios were used to calculate the recoveries.

4. Results and discussion

The chemical structures of fenbufen (I) its metabolites II, IV and ketoprofen V (internal standard) are shown in Fig. 1. During the acidic extraction process, metabolite II is quantitatively transformed into a lactone III in situ and it is this species which is stable and is chromatographed. Fenbufen has no native fluorescence, but is rapidly transformed by UV irradiation to a highly fluorescent species. An increase in fluorescence was also seen with lactone III which has a very weak native fluorescence. Metabolite IV has an appreciable native fluorescence and its response is attenuated upon UV irradiation.

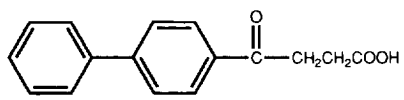
4.1. Post-column photochemical derivatization

4.1.1. Effect of irradiation time on the fluorescence response

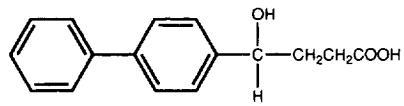
The effect of irradiation time on the fluorescence response was determined by recording the responses of all three analytes at various flow-rates (0.2–1.0 ml/min) which corresponded to various irradiation times using a coil length of 790 cm. From Fig. 2, it can be seen that the irradiation time had a profound effect on the fluorescence response for all three analytes. The optimal fluorescence was obtained for I, III and IV at flow-rates of 0.6, 0.5 and 0.4 ml/min, respectively. A flow-rate of 0.5 ml/min was finally chosen as an appropriate compromise for maximum sensitivity for all three analytes. The fluorescence response of equimolar solutions of the three analytes, fenbufen, lactone III and metabolite IV were 1:1.36:1.25, respectively.

Fig. 3A shows the chromatogram of a serum blank with UV lamp on, Fig. 3B shows the chromatogram of the analytes and internal standard with the UV lamp off, and Fig. 3C shows the same chromatogram with the UV lamp on. The use of an cyanopropyl reversed-phase column enabled the analytes to be separated within a reasonable chromatographic run time using the 0.5 ml/min flow-rate.

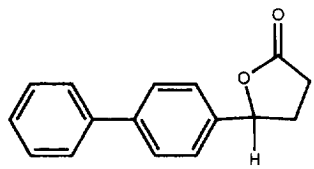
To decrease the sample manipulation and preparation times reported by other methods, a simple SPE procedure was developed for sample cleanup. The C_{18} sorbent was selected because it allowed an excellent elution of fenbufen and its metabolites using methanol as eluent. Elution of the analytes was tried using various solvents including acetonitrile. Methanol was found to contain no interfering peaks and gave high recoveries. The addition of saturated ammonium sulphate solution was found to give



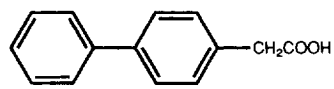
Fenbufen (I)



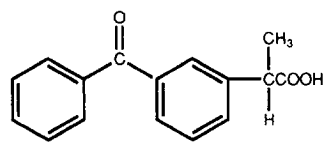
3-(4-biphenylhydroxymethyl)propionic acid (II)



Lactone (III)



4-biphenylacetic acid (IV)



Ketoprofen (V)

Fig. 1. Chemical structures of fenbufen (I), its metabolites II, IV and ketoprofen V (internal standard).

recoveries higher than that obtained with HCl alone and its addition did not produce globules of precipitate which blocked the pores of the SPE column. The mean absolute recoveries using the C_{18} SPE were 87.2±3.1% for fenbufen, 89.1±2.5% for lactone III and 91.4±3.8% for metabolite IV, respectively (n=3). Ketoprofen (V) was selected as the internal standard because it also produced a highly fluorescent species on UV irradiation and behaved similarly through the sample preparation processes with good recovery (84.2±3.6%) (n=3). The limit of detection, based on a signal-to-noise ratio of 5 was determined to be 10 ng/ml for fenbufen and its metabolites.

The calibration curves showed good linearity in the range of 50–375 ng/ml for all three analytes. The coefficients of determination were more than 0.99 for fenbufen, lactone III and IV. Representative linear regression equations obtained for fenbufen and lactone III and metabolite IV were $y=0.006732x-0.0738478$, $y=0.00760855x-0.004565$ and $y=0.0055120x+0.0696$, respectively, where y and x are the drug to internal standard peak-area ratios and concentration of each analyte, respectively. The intra-day precision (n=3) as expressed by R.S.D. and relative error was 4–6.2% and 6.5–7.4% for fenbufen, 3.5–5.8% and 4.9–6.3% for lactone III,

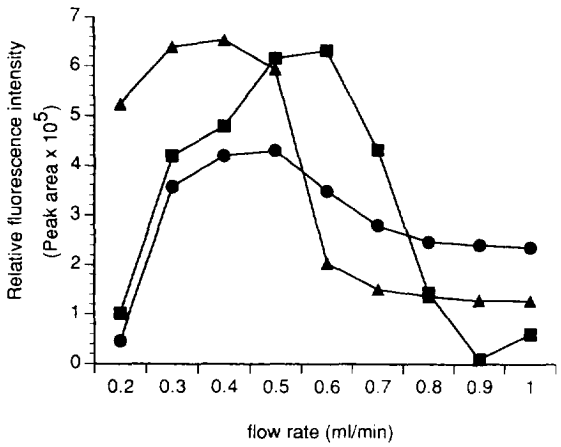


Fig. 2. Peak area response versus flow-rate for fenbufen (I) (■), lactone (III) (●) and metabolite (IV) (▲), after post-column UV irradiation and fluorescence detection.

and 6.0–6.1% and 2.6–5.9% for metabolite IV, respectively. The inter-day precision and accuracy (n=9, over three days) were 4.6–6.5%, and 6.1–5.1% for fenbufen, 5.1–6.4% and 5.0–6.1% for lactone III, and 5.4–7.4% and 5.3–6.0% for metabolite IV, respectively. The detailed data is listed in Table 1.

In summary, a precise, accurate and rapid HPLC method using isocratic conditions and employing solid-phase extraction and post-column UV irradiation has been developed for the analysis of fenbufen and its two major serum metabolites. Since the therapeutic range for fenbufen is in the order of 0.5–10 μg/ml of serum, unknown samples can be diluted easily into the linear range of the method. The method is sensitive to 10 ng/ml of each analyte. The total run time of the isocratic method was less

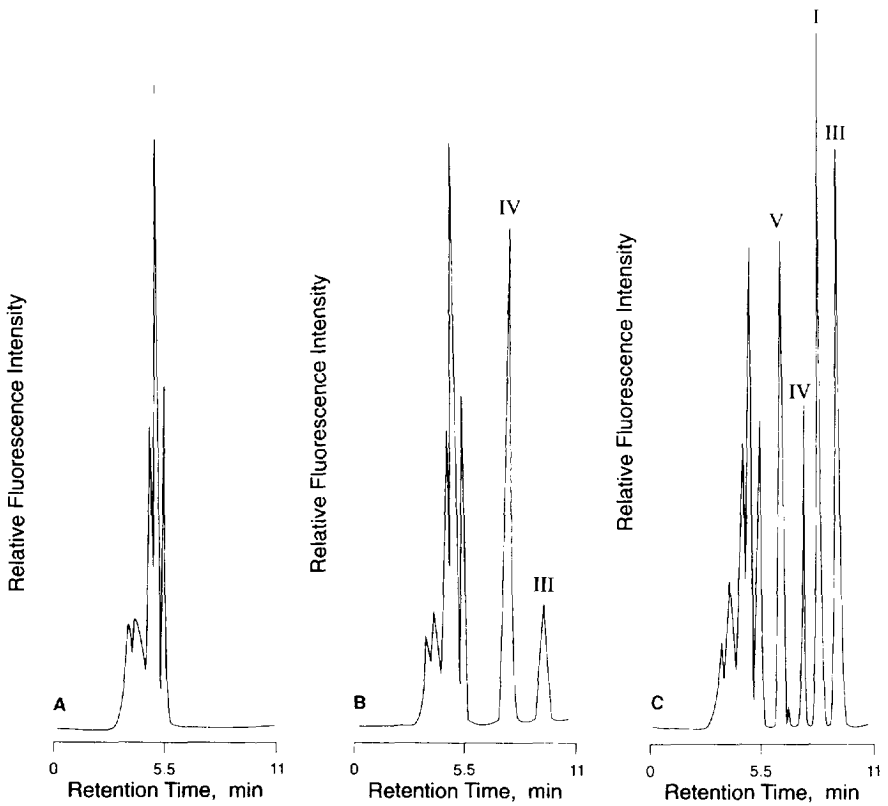


Fig. 3. (A) Representative chromatogram of serum blank with UV lamp on. (B) Representative chromatogram of spiked serum sample containing fenbufen (I), lactone (III), metabolite (IV) and internal standard (V) with UV lamp off. (C) Representative chromatogram of spiked serum sample containing I (220 ng/ml), III (150 ng/ml), IV (100 ng/ml) and V (internal standard, 6 μg/ml) with UV lamp on.

Table 1
Accuracy and precision of serum samples with added fenbufen (I), lactone (III) and metabolite (IV)

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)[a,b]	Relative error (%)	R.S.D. (%)
Intra-day				
I	60	64.45± 2.55	7.4	4.0
	350	327.37±20.44	6.5	6.2
III	60	56.23± 3.24	6.3	5.8
	350	367.07±20.59	4.9	3.5
IV	60	63.56± 3.78	5.9	6.0
	350	340.79±20.59	2.6	6.1
Inter-day				
I	60	63.68± 3.93	6.1	6.5
	350	332.0±16.06	5.1	4.6
III	60	57.01± 3.83	5.0	6.4
	350	371.24±17.86	6.1	5.1
IV	60	56.84± 3.04	5.3	5.4
	350	329.01±25.86	6.0	7.4

[a] Based on $n=3$ for intra-day assay.
[b] Based on $n=9$ for inter-day assay.

than 11 min and is a considerable improvement over existing HPLC methods.

References

[1] B.B. Hansen and S.H. Hansen, J. Chromatogr. B, 658 (1994) 319.
[2] H. Joshua, J. Chromatogr. A, 654 (1993) 247.
[3] I. Urmos, S.M. Benko and I. Klebovich, J. Chromatogr., 617 (1993) 168.
[4] S. Caccia, S. Confalonieri, G. Guiso, S. Celeste and P.P. Marini, J. Chromatogr., 581 (1992) 109.
[5] J. Salamoun, M. Macka, M. Nechvatal, M. Matousek and L. Knesel, J. Chromatogr., 514 (1990) 179.
[6] C. Kikuta and R. Schmid, J. Pharm. Biomed. Anal., 7 (1989) 329.
[7] H. Scholl, K. Schmidt and B. Weber, J. Chromatogr., 416 (1987) 321.
[8] U.A.Th. Brinkman, P.L.M. Welling, G. Devries, A.H.M.T. Scholten and R.W. Frei, J. Chromatogr., 217 (1981) 463.
[9] J. Salamoun and J. Frantisek, J. Chromatogr., 378 (1987) 173.
[10] C.L. Baustain and T.J. Mikkelson, J. Pharm. Biomed. Anal., 4 (1986) 237.
[11] I.S. Lurie and D.A. Cooper and I.S. Krull, J. Chromatogr., 629 (1993) 143.
[12] C.J. Miles and H.R. Moye, Chromatographia, 24 (1987) 628.
[13] C.H. Wolf and R.W. Schmid, J. Liq. Chromatogr., 13 (1990) 2207.
[14] I.S. Krull and X.D. Ding, C. Selavka, and K. Bratin, J. Forensic Sci., 29 (1984) 449.
[15] C.M. Selavka, I.S. Krull, and K. Bratin, J. Pharm. Biomed. Anal., 4 (1986) 83.
[16] C.M. Selavka, I.S. Krull, and I.S. Lurie, J. Chromatogr. Sci., 23 (1985) 499.
[17] W.J. Bachman and J.T. Stewart, LC·GC, 7 (1989) 38.
[18] I.S. Krull and W.R. LaCourse, Trends Anal. Chem., 4 (1985) 118.
[19] J.W. Birks and R.W. Frei, Trends Anal. Chem., 1 (1982) 361.
[20] I.S. Krull and W.R. LaCourse, in I.S. Krull (Editor), Reaction Detection in Liquid Chromatography, Vol. 34, Marcel Dekker, New York, 1986, p. 303.
[21] R. Child, A. Osterberg, S. Sloboda and A. Tomcufcik, J. Pharm Sci., 66 (1977) 466.
[22] G. Cuisinaud, J. Legheand, C. Delkahia and J. Sassard, J. Chromatogr., 148 (1978) 509.
[23] G.E. Vanlear, F.S. Chiccarelli, P.A. Bonenfant and A. Barr, J. Pharm Sci., 67 (1978) 1662.
[24] J.S. Fleitman, S.G. Schulman and J.H. Perrin, J. Chromatogr., 228 (1982) 372.
[25] H. Engelhardt and U.D. Neue, Chromatographia, 15 (1982) 403.