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## Note

# Quantitation of biphenylacetic acid in plasma and synovial fluid by gas chromatography–mass spectrometry–mass spectrometry

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Biphenylacetic acid (BPAA) belongs to the class of drugs collectively known as non-steroidal anti-inflammatory drugs. However, due to potent gastrointestinal irritant effects following oral administration, it is not administered as such but as the prodrug fenbufen. It is thought that the anti-inflammatory effects of fenbufen are due to the metabolic formation of BPAA [1], but again oral administration of fenbufen is not without problems such as gastrointestinal upset. It is believed that non-steroidal anti-inflammatory drugs exert their action at the site of inflammation by interfering with the formation of arachidonic acid metabolites such as prostaglandins [2].

An alternative approach to systemic drug delivery to the site of action, for example the inflamed synovium of a patient with rheumatoid arthritis, is topical application of the drug in a suitable vehicle which aids penetration through the skin into the synovium. This approach may allow sufficient drug to reach the site of action to result in analgesia without having to subject the patient to the undesirable side-effects often associated with oral drug therapy.

In order to investigate the penetration of BPAA into the synovium and to monitor plasma levels of the drug, a technique capable of reliable quantitative determination at low ng/ml concentrations is required. Published assays for BPAA in plasma have used high-performance liquid chromatography (HPLC) [3] and gas chromatography–mass spectrometry (GC–MS) [4], however, both assays have detection limits of 100 ng/ml and neither of these methods appeared to be sensitive enough for our purposes. A modification of the GC–MS method [4] was

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used by Sugawara et al. [5] to determine the plasma and synovial fluid concentrations of BPAA after application of a 3% BPAA gel to the knees of a group of people suffering from a variety of osteo and rheumatic complaints. A detection limit of 5 ng/ml was claimed but no data were supplied to substantiate this, and these authors reported that BPAA levels were below this value in the majority of samples assayed.

Recently the technique of GC–MS–MS has been shown to offer greatly increased sensitivity and selectivity for the detection of specific compounds in complex biological media [6]. We have developed and report here a negative-ion chemical-ionization (NICI) GC–MS–MS method for the determination of BPAA in biological fluids which provides a 500-fold improvement in sensitivity together with far greater selectivity compared with other methods. This assay has been successfully used to monitor plasma and synovial fluid levels of BPAA in patients treated with a topical preparation of BPAA.

## EXPERIMENTAL

BPAA was a gift from Cyanamid International (U.S.A.). All other reagents used were of analytical-reagent grade, all solvents were re-distilled before use. All glassware used for extraction and derivatization procedures was silylated by treatment with a 5% solution of dichlorodimethylsilane in hexane. Deuterated BPAA was synthesised from BPAA and $^2H_2O$ as described below using the method of Whitlam and Vine [7] with some modifications.

### *Internal standard*

To a vigorously stirred mixture of BPAA (100 mg), dichloromethane (10 ml) and aluminium chloride (900 mg), $^2H_2O$ (0.2 ml) was added dropwise over 5 min. The reaction mixture was stirred for 30 min at room temperature and then sufficient $^2H_2O$ was added to decompose the reaction complex. The reaction mixture was centrifuged and the dichloromethane layer was passed through a silica Sep-Pak. The reaction product was eluted from the Sep-Pak with dichloromethane and the product was isolated by evaporation of the solvent. The above process was repeated twice on the product isolated from each previous reaction to give deuterium-labelled BPAA which contained no unlabelled material.

A portion of the third reaction was derivatised to form the pentafluorobenzyl ester and analysis by NICI-MS showed the deuterium content to be: $d_1$ (0.2%); $d_2$ (1.5%); $d_3$ (3.3%); $d_4$ (5.6%); $d_5$ (10.6%); $d_6$ (17.5%); $d_7$ (25.3%); $d_8$ (24.3%); $d_9$ (11.4%) (Fig. 1). The mass and NMR spectra showed that deuteration had taken place exclusively on the aromatic rings.

### *Gas chromatography–mass spectrometry–mass spectrometry*

GC–MS–MS analyses were carried out on a Finnigan TSQ-46 triple stage quadrupole mass spectrometer operating in NICI mode. The BP1 fused-silica bonded-phase capillary column (12 m × 0.2 mm I.D., 0.25 $\mu$m film thickness) was inserted directly into the ion source of the mass spectrometer. Helium (2 ml/min) was the GC carrier gas and methane, the CI reactant gas, was introduced through

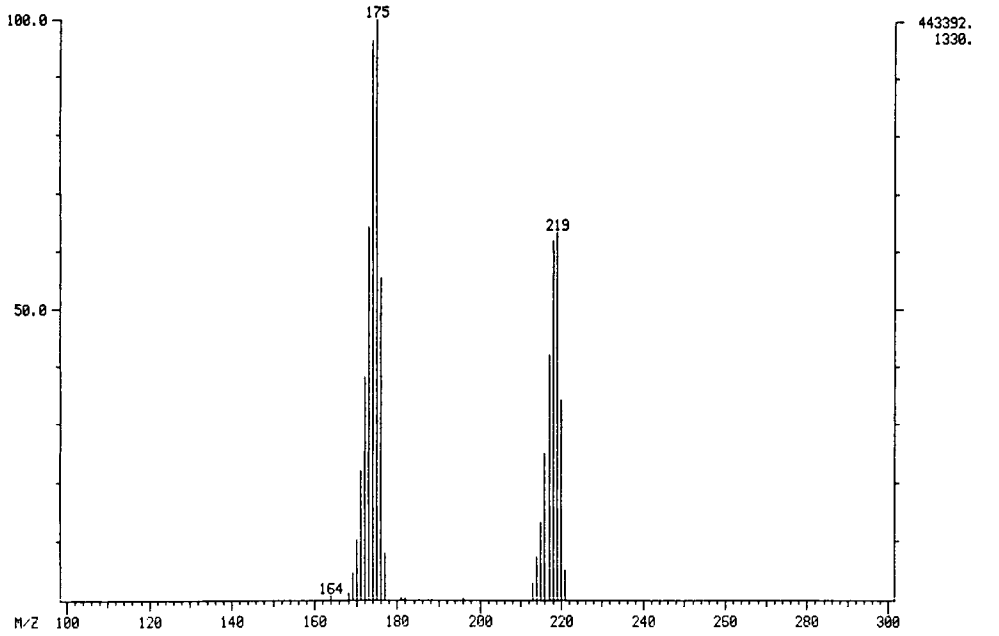


Fig. 1. Methane NICI mass spectrum of the pentafluorobenzyl ester of deuterium-labelled BPAA.

the make-up valve to give a final source pressure of 130 Pa. The ion source and analyser regions of the mass spectrometer were maintained at 100°C and an electron beam energy of 100 eV was used. Collision-activated decomposition (CAD) mass spectra were generated using argon as collision gas at a pressure of 0.2 Pa and the collision energy was 25 eV.

The capillary column was held at 150°C for 1 min following splitless injection of 1-$\mu$l aliquots of extracts and was then programmed to 300°C at 25°/min. The injection port and GC–MS interface temperatures were held at 250 and 300°C, respectively. To minimise contamination of the injector, the septum sweep valve was kept open at all times allowing a helium flow-rate of 5 ml/min across the septum. The split valve was closed immediately prior to the injection and was opened after 1 min. Under these conditions, the pentafluorobenzyl esters of BPAA and BPAA-$d_8$ had retention times of 5.36 and 5.33 min, respectively.

*Extraction and derivatization procedure*

To 1 ml of the appropriate biological fluid was added 0.1 ml of methanol containing nominally 6 ng of BPAA-$d_8$. To this solution was added 1.0 ml of 0.5 *M* hydrochloric acid and the mixture was extracted with 6 ml of freshly redistilled diethyl ether by vortex-mixing for 60 s and was then centrifuged at 700 *g* for 10 min. The ether layer was transfered to an evaporation tube and evaporated to dryness. The residue was dissolved in 0.01 ml of DMF, 0.005 ml of 0.4 *M* tetraethylammonium hydroxide solution and 0.005 ml of pentafluorobenzyl bromide were then added and the mixture was vortex-mixed for 30 s. The solution was allowed to stand for a further 5 min at which time 0.01 ml of water and 0.1 ml of

hexane were added. The tube was vortex-mixed for 60 s and the hexane layer was transferred to a clean silanized tube and a 1-$\mu$l aliquot was assayed by GC–MS–MS.

*Calibration standards*

Standard curves for BPAA in both plasma and synovial fluid were constructed covering the range 0.1–100 ng/ml by spiking 10 ml of plasma or synovial fluid with 0.1 ml of methanol containing the required amount of BPAA, taking 1 ml of this solution and adding nominally 6 ng of BPAA-$d_8$ dissolved in 0.1 ml of methanol. Spiked plasma and synovial fluid along with the appropriate controls were extracted and derivatized as described above. Replicate extractions ($n=4$ or 5 at 0.1 ng/ml and $n=8$ at 100 ng/ml) were performed at the highest and lowest points on the calibration curve to give a measure of the precision of the procedure and duplicate extractions were performed at the intermediate concentrations. New standard curves were constructed on each day samples were to be analysed.

## RESULTS AND DISCUSSION

The NICI mass spectrum of the pentafluorobenzyl ester of BPAA (BPAA-PFB) contained ions at *m/z* 211 and 167 (Fig. 2) corresponding to the carboxylate anion of BPAA and the biphenylmethyl anion, respectively. The relative abundance of ions produced by NICI is often greatly influenced by ion source temperature and in this case a temperature of 100°C was found to give the best compromise of high abundance of *m/z* 211 without excessive contamination of the ion source.

Full-scan mass spectra were acquired for the CAD daughters of *m/z* 211 at several collision energies, the optimum collision energy was found to be 25 eV and gave a spectrum containing *m/z* 167 as base peak. For the deuterated-BPAA internal standard, *m/z* 219, which corresponds to BPAA-$d_8$, was selected as the parent ion. Collision activated decomposition of this ion under the same conditions used for *m/z* 211 resulted in a daughter ion at *m/z* 175. BPAA was analysed by monitoring the daughter ion of *m/z* 211 at *m/z* 167 for BPAA-PFB and the corresponding daughter ion of *m/z* 219 at *m/z* 175 for BPAA-$d_8$-PFB. Peak areas were measured automatically by the data system and calibration curves were constructed and BPAA concentrations calculated using the Finnigan TSQ data system quantitative programs.

Calibration curve data for BPAA in both plasma and synovial fluid showed excellent linearity (equation of a typical regression line $y=mx+c$, where $m=0.155$, $c=-0.0486$ and $r=0.9999$) and BPAA at a concentration of 0.1 ng/ml could be detected with a coefficient of variation of 5.6% ($n=5$) in plasma and 8.7% ($n=4$) in synovial fluid. This was equivalent to an on-column injection of approximately 1 pg of BPAA. At a concentration of 100 ng/ml, the coefficients of variation were 2.1% ($n=8$) in plasma and 1.1% ($n=8$) in synovial fluid. The estimated detection limit for this assay was approximately 0.01 ng/ml. The method showed excellent day-to-day reproducibility over a six-day period. Standard samples of BPAA, analysed each day, had coefficients of variation of 1.9% ($n=6$) at

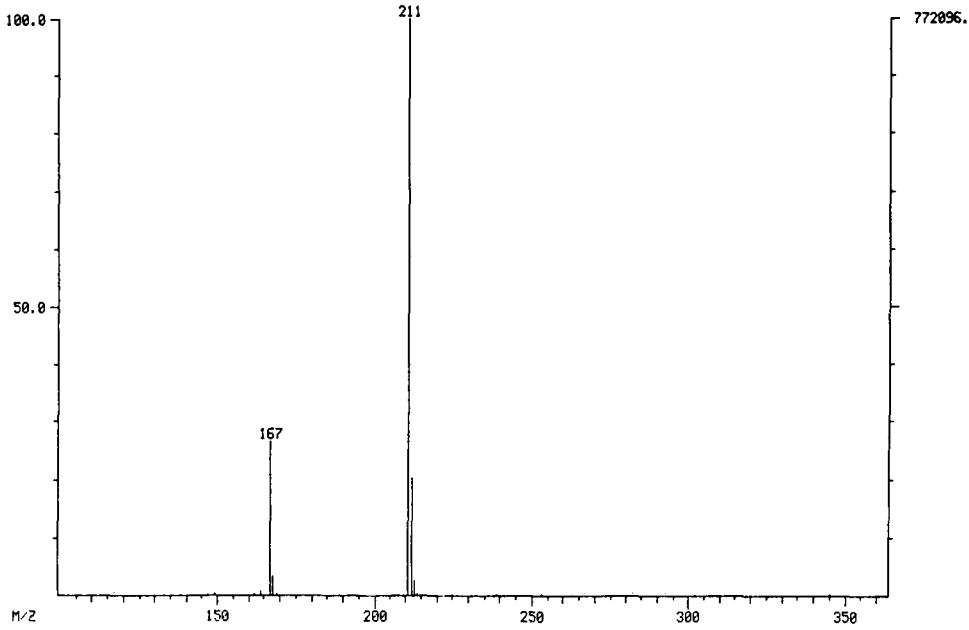


Fig. 2. Methane NICI mass spectrum of the pentafluorobenzyl ester of BPAA.

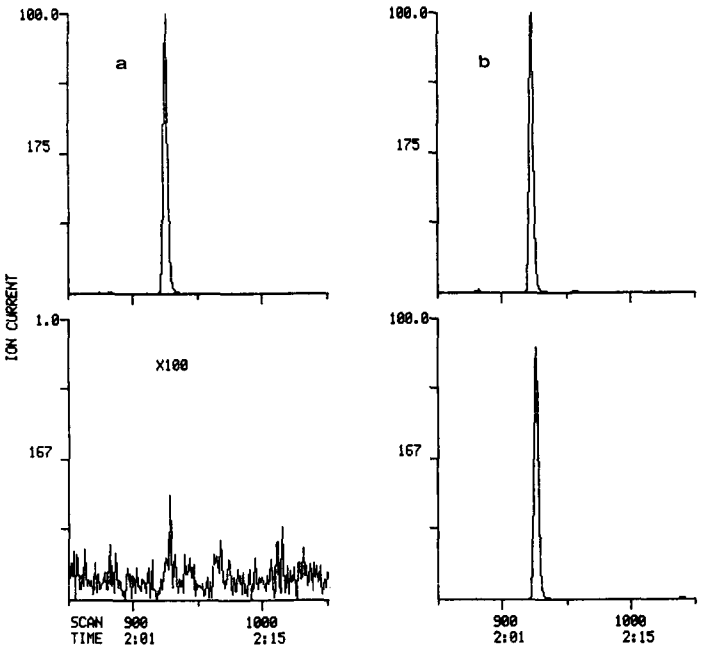


Fig. 3. Ion chromatograms $m/z$ 167 (BPAA) and 175 (BPAA-$d_8$) from the synovial fluid of a patient (a) before treatment and (b) at 4 h after treatment with the BPAA gel; calculated BPAA concentration 7.3 ng/ml.

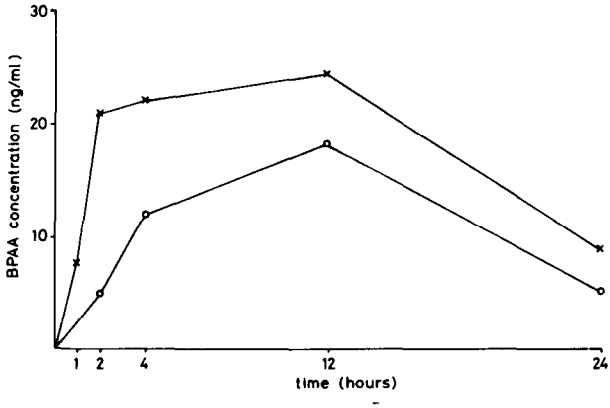


Fig. 4. Plasma (×) and synovial fluid (○) versus time curves for a patient treated with 3 g of 3% BPAA gel.

a concentration of 2 ng/ml and 1.7% ($n=6$) at a concentration of 50 ng/ml. Approximately 30 to 40 samples could be analysed in a day.

This method has been successfully used to measure concentrations of BPAA in the plasma and synovial fluid of rheumatoid arthritis patients to whom 3 g of a 3% BPAA gel had been applied to the knee. Fig. 3a is a chromatogram obtained from the synovial fluid of a patient before treatment and Fig. 3b from the same patient 4 h after application of the gel, at which time the BPAA concentration was calculated to be 7.3 ng/ml. Fig. 4 shows the plasma and contralateral knee synovial fluid concentration versus time curve for a patient who was treated as described above. A detailed description of BPAA levels in the plasma and synovial fluid of fourteen patients treated with the BPAA gel will be reported elsewhere.

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