ELSEVIER

Journal of Chromatography B, 654 (1994) 293–296

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

High-performance liquid chromatographic determination of iophenoxic acid in serum

Ainsley Jones

Central Science Laboratory, Ministry of Agriculture, Fisheries and Food, London Road, Slough, SL3 7HJ, UK

(First received July 30th, 1993; revised manuscript received January 10th, 1994)

Abstract

Iophenoxic acid (IPA), a marker used to investigate the feeding behaviour of bait-consuming animals has previously been indirectly determined by measuring protein-bound iodine levels in serum or plasma. For the first time a method is reported for the direct determination of IPA in biological fluids. IPA was determined in de-proteinized serum by high-performance liquid chromatography (HPLC) on a C_{18} column with a mobile phase of acetonitrile–water. Isocratic and gradient systems are described with limits of detection of 0.2 μg/ml (isocratic) and 0.05 μg/ml (gradient). Recoveries from fox serum were 85% at 0.5 μg/ml, 95% at 5 μg/ml and 91% at 50 μg/ml.

1. Introduction

Iophenoxic acid (α-ethyl-3-hydroxy-2,4,6-triiodobenzenepropanoic acid) has been clinically employed as a radio-opaque medium for visualisation of the gallbladder and was sold under the trade name Teridax. During the 1950s it was realised that because of its high affinity for serum albumin iophenoxic acid (IPA) caused gross and extremely prolonged elevation of serum iodine levels [1]. Measurement of serum iodine was, at the time, one of the most accurate indicators of thyroid function but administration of a single dose of IPA invalidated the results for up to 30 years [2]; clinical use of IPA was thus discontinued [3].

However IPA has been useful to biologists in bait acceptance studies of wild animals as a serum marker, *i.e.* a low toxicity compound which can be detected in the serum of animals that have consumed IPA-marked baits [4–8]. In all reported studies IPA was not directly determined, instead its presence was inferred from raised levels in the serum or plasma of protein-bound iodine (PBI), measured by variations of the method of Riley and Gochman [9]. In this laboratory we have used baits of gelatin-stiffened mechanically recovered meat containing 40 mg IPA to study bait uptake by the red fox (*Vulpes vulpes*). Background PBI levels of 2–8 μg/100 ml have been reported for this species [4,5] although the true variation may be greater than this. We wished to be able to detect IPA in fox serum for at least 8 weeks after bait consumption. The results of Saunders *et al.* [10] with captive foxes suggested that, 8 weeks after ingesting 40 mg of IPA, serum PBI levels may be less than 7 μg/100 ml above the baseline level. Our trials involve wild foxes for which the background PBI of each individual is not known. It would therefore be very difficult to distinguish, 8 weeks after bait consumption, between an IPA-

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(94)00020-6

induced elevated PBI level and a background PBI level toward the upper end of the normal range. We thus required a specific, quantitative method that could determine IPA in serum at 0.1 μg/ml (equivalent to 7 μg/100 ml PBI) or lower.

Only one procedure for IPA analysis has been reported [11]. The authors described the determination of IPA by GC–ECD as its trimethylsilyl derivative but did not investigate the quantitative aspects of the method and did not apply it to biological fluids. HPLC has been used for determination of other iodinated radio-opaque chemicals including iothalamate [12–15] and iopentol [16]. This paper presents an HPLC method for the direct determination of IPA in serum.

2. Experimental

2.1. Apparatus and materials

The HPLC system consisted of two Waters 510 pumps, a Waters WISP 712 autosampler (Millipore, Watford, UK), and an Applied Biosystems 785 variable wavelength UV detector operated at 229 nm. The column (250 × 4.6 mm I.D., stainless steel) was of 5-μm Spherisorb ODS2 (Jones Chromatography, Hengoed, UK) and was protected with a guard column (10 × 4.6 mm I.D.) of Spherisorb 5 μm ODS1. The detector signal (1 V/AU) was sent to a NEC 386SX PC and data was acquired and analyzed using Waters Baseline software. Centrifugation was performed on a Heraeus Minifuge 2 (Brentwood, UK).

Methanol (HPLC grade) was from Rathburn (Walkerburn, UK). Water was double-distilled in this laboratory. Acetic acid (glacial), sulphuric acid (sp. gr. 1.84) and sodium tungstate 2-hydrate were of AnalaR grade (BDH, Poole, UK). Pyrex glassware was used throughout.

2.2. Extraction

To 0.4 ml of fox serum in a polystyrene centrifuge tube was added 0.8 ml of sulphuric acid solution (0.33 *M*), 0.8 ml of sodium tungstate 2-hydrate solution (10% w/v in water) and 3 ml of methanol. The solution was allowed to stand at room temperature (*ca.* 22°C) for 15 min and centrifuged at 1800 *g* for 20 min. The supernatant was transferred to a volumetric flask and the volume adjusted to exactly 5 ml with methanol–water (60:40, v/v).

2.3. High-performance liquid chromatography

For the gradient system the mobile phases consisted of solvent A, which was water containing 0.25% (v/v) acetic acid, and solvent B, which was acetonitrile containing 0.125% (v/v) acetic acid. The initial conditions were 75% A and 25% B. The composition was changed linearly to 40% A and 60% B after 0.3 min and then changed linearly to 100% B after 8 min. At 10 min the composition was changed over a period of 1 min to the initial conditions and equilibrated for 4 min. The mobile phase for the isocratic system was acetonitrile–water (60:40, v/v) containing 0.25% acetic acid.

Injections of 300 μl were made via the autosampler onto the HPLC column. Quantitation was by means of calibration curves constructed from peak-height measurements using standard solutions in methanol–water (60:40, v/v). These were prepared daily in the range 0.005 to 5 μg/ml from a stock solution of 500 μg/ml in methanol which was prepared fortnightly and stored at 4°C.

2.4. Recoveries and inter-assay precision

Recovery experiments at three levels were performed by adding 25 μl of the appropriate standard solution to 0.4 ml of serum which was then shaken and allowed to equilibrate for 10 min before being extracted as described above. One determination was made at each level for six days. Recoveries were measured using the gradient HPLC system described above and the results are shown in Table 1.

2.5. Intra-assay precision

The intra-assay precision was calculated from six simultaneous determinations using the gra-

dient system, of a serum sample from a fox that had consumed IPA six weeks previously.

3. Results and discussion

The gradient system gave rise to sharp, nearly symmetrical peaks and the limit of reliable detection in serum was 0.05 μg/ml. A 6-point calibration curve of amount injected against peak height was linear up to 10 μg/ml with a correlation coefficient of 0.9995. The slope was $1.410 \cdot 10^5$ with an S.D. of 2369 and the y-intercept was 10 197. Typical chromatograms are shown in Fig. 1. Fig. 1a shows an IPA standard, Fig. 1b is from a serum sample spiked with 50 μg/ml IPA and Fig. 1c is from a blank serum sample and demonstrates that there are no significant interfering peaks.

The isocratic system gave broader IPA peaks with a lower height-to-area ratio which were less well separated from low level serum constituents. Consequently the detection limit in serum of 0.2 μg/ml was higher than with the gradient system. The calibration curve was again linear up to 10 μg/ml with a slope of $7.867 \cdot 10^4$ (S.D. = 1468) and a y-intercept of 2035. Fig. 2 shows a typical chromatogram from the isocratic system.

Due to our need to detect IPA at less than 0.1 μg/ml in this study it was decided to use the gradient system for evaluation of accuracy and precision. However the simpler isocratic system should prove adequate for many feeding studies in which IPA is used.

A number of procedures to dissociate IPA from serum albumin were investigated. Methods involving precipitation of albumin with organic solvents such as methanol, acetonitrile and acetone were unsuccessful. Organic solvents do not effect complete precipitation of proteins from serum or plasma [17] and IPA has such a high affinity for albumin that it appears necessary to precipitate 100% of the albumin present to ensure adequate recoveries. The tungstate-H_2SO_4 procedure has been reported to be extremely effective in precipitating proteins [17] but initially proved unsuitable for this work since IPA is insoluble in aqueous solutions below pH 8. A combination of tungstate-H_2SO_4 and methanol gave acceptable recoveries.

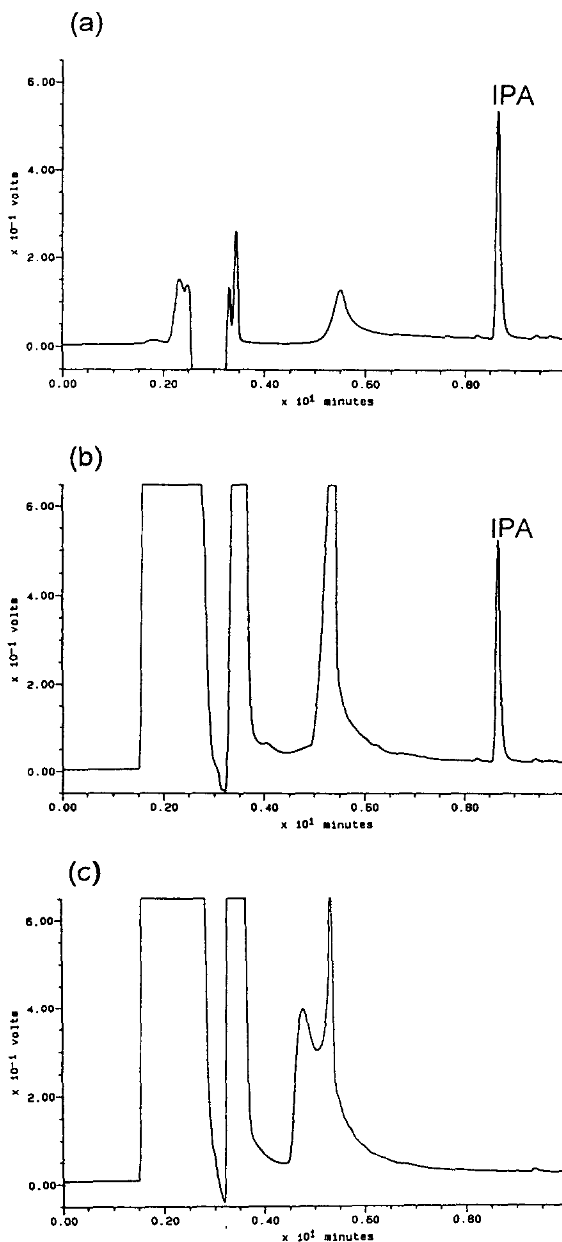

Fig. 1. Typical high-performance liquid chromatograms from gradient sytem. (a) IPA standard 1.2 μg; (b) fox serum spiked with 50 μg/ml IPA; (c) blank control fox serum.

The intra-assay precision had a coefficient of variation of 6.2%. The mean concentration was 0.329 μg/ml.

Together with the results shown in Table 1 this

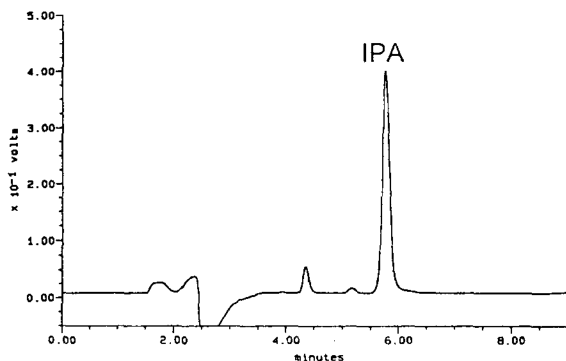

Fig. 2. Typical chromatogram from isocratic sytem, IPA standard 1.8 μg.

demonstrates that the accuracy and precision of the method are adequate for use in feeding behaviour studies. The use of an internal standard would have been desirable to improve the precision of the results but this would require a similar compound with comparable affinity for serum albumin. Other iodinated radio-opaque chemicals were considered but these are much less strongly bound to albumin. To date it has not proved possible to find a compound suitable for use as an internal standard.

This is the first reported method for direct determination of iophenoxic acid. The procedure is simple and allows for reliable determination of IPA from 0.05–50 μg/ml in serum. It has already been used in this laboratory in studies involving the red fox and found to be superior to PBI determination for unequivocal identification of IPA, especially at low levels. These results will be reported elsewhere.

Table 1
Recoveries of iophenoxic acid added to vulpine blood serum

IPA added (mg/ml)	Recovery (%)	Coefficient of variation (%)
0.5	85	9.3
5.0	95	5.7
50	91	4.8

Average of six replicate determinations, one determination at each level for six days.

4. References

[1] E.B. Astwood, *Trans. Assoc. Am. Physicians.*, 70 (1957) 183.
[2] R.R. Hall and W.P. Vanderlaan, *J. Am. Med. Assoc.*, 177 (1961) 648.
[3] R. Shapiro and E. Man, *J. Am. Med. Assoc.*, 173 (1960) 1352.
[4] G.E. Larson, P.J. Savarie and I. Okuno, *J. Wildl. Manage.*, 45 (1981) 1073.
[5] G.M. Baer, *J. Wildl. Manage.*, 49 (1985) 49.
[6] E.H. Follman, P.J. Savarie, D.G. Ritter and G.M. Baer, *J. Wildl. Dis.*, 23 (1987) 709.
[7] J. Hadidian, S.R. Jenkins, D.H. Johnston, P.J. Savarie and G.M. Baer, *J. Wildl. Dis.*, 25 (1989) 1.
[8] W.J. Trewella, S.J. Harris, G.C. Smith and A.K. Nadian, *J. Appl. Ecol.*, 28 (1991) 454.
[9] M. Riley and M. Gochman, Technicon Co. Bulletin 62, Technicon Instruments Corp., New York, 1964.
[10] G. Saunders, S.J. Harris and C.T. Eason, *Wildl. Res.*, 20 (1993) 297.
[11] E.T. Backer and V.J. Pileggi, *J. Chromatogr.*, 36 (1968) 351.
[12] S. Boschi and B. Marchesini, *J. Chromatogr.*, 224 (1981) 139.
[13] T. Prueksaritanont, M.-L. Chen and W.L. Chou, *J. Chromatogr.*, 306 (1984) 89.
[14] M.M. Reidenberg, B.J. Lorenzo, D.E.Drayer, J. Kluger, T. Nestor, J.C. Reigner, B.A. Kowal and I. Bekersky, *Ther. Drug Monitor.*, 10 (1988) 434.
[15] F. Gaspari, L. Mainardi, P. Ruggenenti and G. Remuzzi, *J. Chromatogr.*, 570 (1991) 435.
[16] A.T. Andersen, P.B. Jacobsen and K.E. Rasmussen, *J. Chromatogr.*, 575 (1992) 93.
[17] J. Blanchard, *J. Chromatogr.*, 226 (1981) 455.