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Radioimmunoassay for ubenimex in human serum

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Abstract: Anti-ubenimex antibody was produced by immunization of rabbits with ubenimex–bovine serum albumin (BSA) conjugate which was synthesized by coupling ubenimex directly to BSA using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The resulting antibody exhibited little cross-reactivity with *p*-hydroxyubenimex or (2*S*,3*R*)AHPA, (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoic acid present in human serum as metabolites. Ubenimex in human serum could be measured selectively without prior drug extraction and purification. The standard curve prepared using the present assay method gave good linearity in the range 0.25–25 ng per assay tube. The detection limit was 125 pg per assay tube. The concentrations of ubenimex in human serum determined by the present method were in good agreement with those obtained by GC–SIM. The present method with its high sensitivity and specificity may be a valuable tool for the study of the pharmacokinetics of ubenimex.

Keywords: *Ubenimex; radioimmunoassay; metabolites; pharmacokinetics.*

Introduction

Ubenimex (formerly known as Bestatin) is *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine]; it is isolated from the fermentation broth of *Streptomyces olivoleticuli*. The drug inhibits aminopeptidase B and leucine aminopeptidase, enhances delayed type hypersensitivity and is a promising new type of immunomodulator [1, 2]. To investigate the pharmacokinetics of ubenimex, a highly sensitive and specific method for the determination of ubenimex in biological fluids is required. Previously, a method has been reported for the simultaneous determination of ubenimex and *p*-hydroxyubenimex in human serum by GC–SIM using deuterated variants as internal standards [3]. Although GC–SIM is the most sensitive and specific method, it requires tedious procedures such extraction and purification before subjection to GC–SIM.

The present paper describes a new simple and sensitive method for the determination of ubenimex in human serum by radioimmunoassay.

Materials and Methods

Reagents

Ubenimex, (2*S*,3*R*) AHPA and their stereoisomers were synthesized in the authors' laboratories. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St Louis, MO, USA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ethyl-CDI) from Tokyo Kasei Co. (Tokyo, Japan). ^{3}H-Ubenimex, *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-(2′-^{3}H)phenylbutanoyl-L-leucine, with a specific radioactivity of 15 mCi mg^{-1} (5.55×10^8 Bq mg^{-1}) was synthesized in the authors' laboratories [4]. Its chemical and radiochemical purities were found to be more than 99% by thin-layer chromatography and high-performance liquid chromatography using a radioisotope detector.

Preparation of immunizing conjugate

Ubenimex was directly conjugated with BSA using ethyl-CDI by the method of Gharib [5]. Fifty milligrams of BSA and 30 mg of ethyl-CDI were dissolved in 25 ml of distilled water. To this solution, 20 mg of ubenimex containing 7.6 μCi (2.81×10^5 Bq) of ^{3}H-ubenimex dissolved in 5 ml of dimethyl sulphoxide was added with stirring for 30 min at room temperature; the reaction mixture was kept under the same conditions for a further 10 min. An additional 10 mg of ethyl-CDI was added. During this reaction, the pH was kept constant at 5.5 by the addition of 0.1 M HCl or NaOH; the reaction mixture was then kept at room temperature with stirring for 18 h. The reaction mixture was dialysed against distilled water. The dialysate was lyophilized to yield

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45.5 mg of the conjugate. The molar ratio of ubenimex incorporated into BSA was 8.7:1.

Preparation of antisera

A 2 mg volume of the ubenimex–BSA conjugate dissolved in 1 ml of physiological saline was emulsified in an equal volume of complete Freund's adjuvant and injected every 3 weeks into rabbits at multiple subcutaneous sites. The animals were bled from the ear vein 3–5 days after the final booster injection. The serum collected was stored at −20°C.

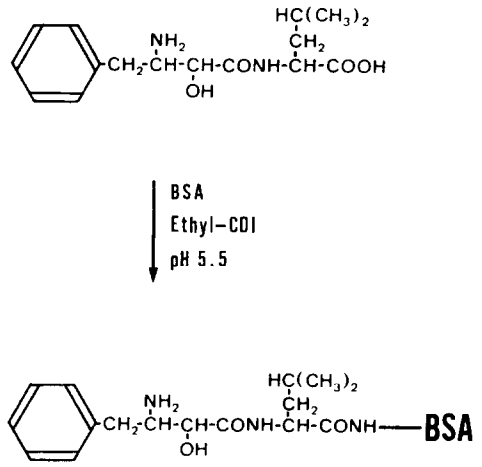
Assay

The buffer solution used throughout this procedure including the preparation of dilutions of the standard solution, tracer and antiserum was 0.05 M phosphate buffer (pH 7.3) containing 0.1% of NaN_3, 0.1% of gelatin and 0.9% of NaCl. Samples were assayed in triplicate. For construction of the ubenimex standard curve, 100 μl of drug-free control serum solution, 100 μl of ^{3}H-ubenimex solution (about 10,000 dpm) and 100 μl of antiserum solution (1:200) were added to 100 μl of the ubenimex standard solution. The buffer solution was used to adjust the final volume to 500 μl. For the assay of unknown samples, 100 μl of diluted sample solution was used in place of the ubenimex standard solution. The reaction mixture was incubated overnight at 5°C. Then 500 μl of a 2% aqueous dispersion of charcoal coated with 1% dextran T 70 was added. After further incubation for 15 min at 5°C this mixture was centrifuged for 15 min at 3000 rpm. The resulting supernatant was decanted into a scintillation vial and dissolved in 3 ml of Atomlight liquid scintillator. The radioactivity was counted by a liquid scintillation counter (Aloka LSC-753, Tokyo, Japan).

Results and Discussion

It was necessary to develop a sensitive and simple radioimmunoassay for the determination of ubenimex in human serum in order to facilitate the study of its pharmacokinetics. A critical step in the development of the assay for ubenimex was the preparation of the immunogen. Ubenimex is a small dipeptide molecule (MW = 308.21) and possesses two reactive residues. This structural characteristic makes it difficult to prepare an effective immunogen since commonly used protein coupling conditions with glutaraldehyde and carbodiimide tend to yield unexpected polymers.

Therefore, the method of Gharib [5] was used to bind a carboxylic acid residue of ubenimex to BSA. By keeping the pH at 5.5 during this reaction with ethyl-CDI as a coupling reagent, a homogeneous ubenimex–BSA conjugate could be obtained without the formation of ubeniumex polymer (Scheme 1). Antisera were obtained from the two rabbits immunized with the ubenimex–BSA conjugate. The titre of antiserum reached a maximum after 4 months and remained constant thereafter. The titre used in this assay was 1:200.



Scheme 1
Synthesis of ubenimex–BSA conjugate.

The cross-reactivity of antiserum obtained from rabbits was determined by measuring the 50% inhibition of ^{3}H-ubenimex–antibody binding using ubenimex, *p*-hydroxyubenimex and (2*S*,3*R*)AHPA. Experiments on the relative cross-reactivity of ubenimex to its related compounds showed little cross-reactivity (0.4%) with *p*-hydroxyubenimex and (2*S*,3*R*)-AHPA, the major metabolites in human serum, and negligible cross-reactivity with other analogues (Table 1). These results demonstrate that this antiserum can be used to determine ubenimex without interference from major metabolites such as *p*-hydroxyubenimex and (2*S*,3*R*)AHPA. A standard curve was prepared using ^{3}H-ubenimex as a tracer (Fig. 1). Good linearity was obtained in the range 0.25–25 ng per assay tube. The lower detection limit was 125 pg per assay tube. The recovery of ubenimex by the present method was 92.8–101.5% irrespective of the concentration of

Table 1
Cross-reactivities of structurally related analogues of ubenimex with ubenimex–antiserum

Compounds	IC_{50}	Cross reactivity (%)
(2*S*,3*R*,*S*)Ubenimex	1.4 ng	100
(2*S*,3*R*,*S*)*p*-Hydroxyubenimex	350.0 ng	0.4
(2*S*,3*R*)AHPA	350.0 ng	0.4
(2*S*,3*S*,*S*)Ubenimex	>10 μg	<0.014
(2*R*,3*R*,*S*)Ubenimex	>10 μg	<0.014
(2*R*,3*S*)AHPA	>10 μg	<0.014
(2*S*,3*S*)AHPA	>10 μg	<0.014
(2*R*,3*R*)AHPA	>10 μg	<0.014

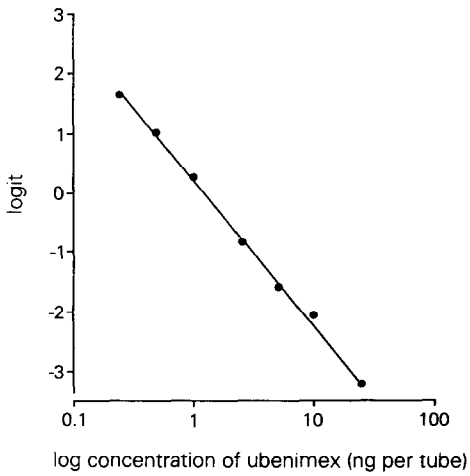


Figure 1
Ubenimex standard curve obtained by the present method.

ubenimex in human serum; the relative standard deviation within the assay was 4.5–14.5% (Table 2).

The present method was applied to the determination of serum levels of ubenimex and the results obtained were compared with those of GC–SIM analysis. A 30 mg volume of ubenimex was administered to each of six healthy volunteers in a single oral dose. Blood samples were taken 0.25, 0.5, 1, 2, 3, 4, 6 and 8 h after administration. The serum levels of ubenimex determined by both GC–SIM and the present method are shown in Fig. 2. The

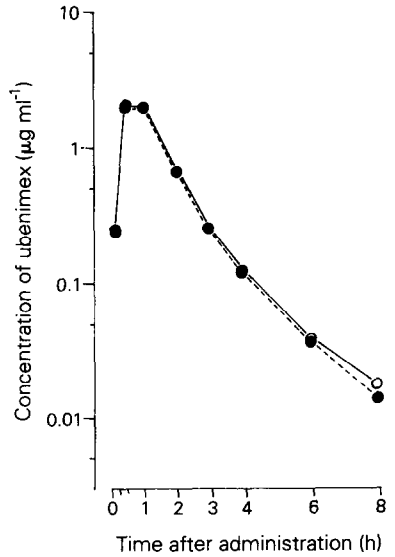


Figure 2
Time course of ubenimex concentration in human serum after oral administration of 30 mg ubenimex to six male volunteers measured by the present method (--●--) and GC–SIM (—●—).

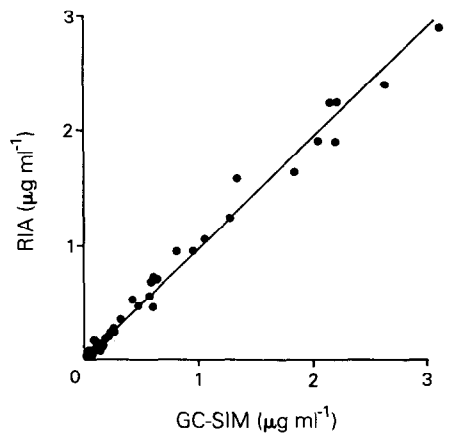


Figure 3
Correlation between ubenimex concentration in human serum quantified by means of the present method and GC–SIM.

data for each time interval were submitted to *t*-test statistical analysis. There was no significance between the results obtained by these two methods. The correlation between the

Table 2
The recovery of ubenimex from drug-supplemented human serum

Ubenimex (ng per tube)	*n*	Found (ng per tube)	Recovery (%)	Relative standard deviation (%)
0.5	4	0.51 ± 0.07	101.5 ± 14.7	14.5
1.0	5	0.99 ± 0.05	99.4 ± 4.5	4.5
2.5	5	2.42 ± 0.14	96.8 ± 5.8	6.0
10.0	5	9.28 ± 1.03	92.8 ± 10.3	11.1

concentration of ubenimex in serum determined by the present method and GC–SIM is shown in Fig. 3.

The regression equation and correlation coefficient were $y = 0.97148x + 0.01473$ and $r = 0.989$, respectively, where x was the analytical result by GC–SIM and y was that by the present method.

In conclusion, the method for ubenimex described in this study is sensitive, specific and reliable. It is useful for long-term clinical pharmacokinetic studies, especially where only small sample volumes are available.

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