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DETERMINATION OF FEZOLAMINE AND ITS DESMETHYL METABOLITE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INTRAVENOUS PHARMACOKINETICS IN THE BEAGLE HOUND

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SUMMARY

Sensitive and selective high-performance liquid chromatographic methods for the quantitation of the experimental antidepressant fezolamine and its desmethyl metabolite in plasma and urine have been developed. Both assays are linear between 0 and 500 ng/ml in both plasma and urine and have calculated minimum quantifiable levels of less than 10 ng/ml. Statistical evaluation of analytical parameters under single-blind conditions demonstrated an overall precision within ±4% of nominal for both compounds in either biological medium. The overall accuracies of the assays were within ±5% of nominal values in urine and ±10% of nominal values in plasma.

Following intravenous administration of fezolamine fumarate to beagle hounds, a biexponential decline in drug plasma levels was observed with the first phase having a half-life of about 11 min and the second phase about 2.6 h. Peak plasma levels of the metabolite were observed at 2 h. Recovery of the parent drug in urine was less than 5% of the administered dose and less than 1% for the desmethyl metabolite.

INTRODUCTION

Fezolamine (Fig. 1, I) is an experimental antidepressant drug currently undergoing clinical evaluation. Its diphenylpyrazole structure differs from the fused three-ring system of the typical dibenzazepines, but does possess the tertiary amine side-chain of known effective antidepressants. In animal models, the drug exhibits antireserpine and antitetrabenazine activities similar to those of imipramine, but the undesirable anticholinergic, sedative, and antihistaminic effects associated with dibenzazepines were considerably reduced or non-existant [1].
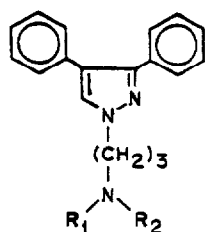
I. Fezolamine
    R_1 = CH$_3$
    R_2 = CH$_3$
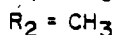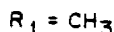
II. Desmethyl Fezolamine
    R_1 = CH$_3$
    R_2 = H
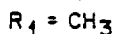
III. Di desmethyl Fezolamine
    R_1 = H
    R_2 = H
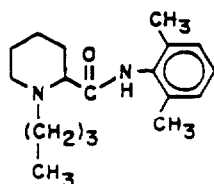
IV. Internal Standard
    Bupivacaine

Fig. 1. Structure of fezolamine and its metabolites.

In the present report, the analytical methods for the simultaneous quantitation of the drug and its desmethyl metabolite (Fig. 1, II) in both urine and plasma are described. Statistical evaluation of relevant analytical parameters and a demonstrated use of the method in a preliminary determination of drug pharmacokinetics in beagle dogs are included.

EXPERIMENTAL

Reagents

Fezolamine (Fig. 1, I), the desmethyl metabolite (Fig. 1, II), and the internal standard, Bupivacaine® (Fig. 1, IV), were synthesized at Sterling-Winthrop Research Institute and used without further purification. Acetonitrile was HPLC grade, and water was treated with an Organicpure® system. Anhydrous diethyl ether, glacial acetic acid, and monobasic sodium phosphate were reagent quality or better. All glassware was acid-washed and rinsed in methanol. Sample tubes were silanized with dimethyldichlorosilane prior to use.

Preparation of chromatographic standards

Standards for the calibration of the chromatographic system were prepared to final concentrations of 0, 50, 75, 100, 250, and 500 ng/ml by the addition of aliquots of a serially diluted stock solution containing both fezolamine and its desmethyl metabolite to 1.0 ml of human urine or 1.0 ml of human plasma (oxalate anticoagulant). Duplicate standards were freshly prepared at each concentration for each analytical run.

Preparation of samples

Samples used for the evaluation of the analytical method were prepared under single-blind conditions by the addition of aliquots of the stock solution

to 1.0 ml of the appropriate biological medium. Two sets of samples were prepared, each set consisting of triplicate samples prepared at a minimum of five concentrations. One set of samples was analyzed immediately after analysis and the second set was analyzed after storage in the laboratory freezer (−4°C) for a minimum of four days.

Extraction

To each of the plasma samples or standards were added 50 μl of a 100 μg/ml internal standard solution and 4 ml of water. Denaturation of plasma proteins was accomplished by the addition of 100 μl of 1 M hydrochloric acid and 10 ml of diethyl ether with thorough mixing. Following centrifugation, the ether layer was discarded and the aqueous layer mixed with 100 μl of 10 M sodium hydroxide and extracted with two 5-ml volumes of diethyl ether. The ether extracts were combined and evaporated to dryness at approximately 40°C under a stream of nitrogen. The residue was dissolved in 300 μl of mobile phase (see below) in preparation for chromatographic analysis.

To each of the urine samples or standards were added 100 μl of the internal standard solution and 0.5 ml of 0.2 M sodium carbonate buffer, pH 10.8. This was followed by extraction with two 5-ml volumes of diethyl ether. The ether extracts were combined and back-extracted with 0.4 ml of 0.1 M hydrochloric acid. The aqueous layer was placed briefly in a 60°C water bath under a stream of nitrogen to remove traces of diethyl ether and was then ready for injection into the chromatographic system.

Chromatography

The modular high-performance liquid chromatographic (HPLC) system consisted of a Milton Roy minipump, a Waters WISP automated injector, a Waters μBondapak C_{18} (10 μm) analytical column (30 × 0.39 cm) and a Corasil C_{18} (37—50 μm) pre-column (2.3 × 0.39 cm). Chromatographic peaks were detected with a Waters 440 ultraviolet detector fitted with a 254-nm filter. In general, 100-μl samples were injected into the system. Raw chromatographic data were collected by a Hewlett-Packard 3356 Laboratory Automation System (LAS).

Statistical analysis

Concentrations of fezolamine and its desmethyl metabolite were determined from a linear least-squares regression of the peak-height ratio (drug or metabolite peak height to internal standard peak height) as a function of concentration. Concentrations were determined directly from the raw data files by the Hewlett-Packard 3356 system. Also calculated was the minimum quantifiable level (MQL), defined as the concentration at which the lower 80% confidence interval of the regression line just encompasses zero [2].

Estimates of recovery were made by preparing samples in triplicate at five concentrations in the appropriate biological medium. The samples were then extracted as described above. Recovered concentrations were determined from a regression line obtained by injection of serially diluted drug stock solutions. The percent recovery is reported as the overall mean value

for all concentrations. Estimates of the internal standard recovery were made at a single concentration and based upon the mean peak heights of the extracted samples relative to those of the stock solution.

The precision of the assay was defined as the overall standard deviation of the mean percent differences from nominal concentrations. The accuracy of the assay is defined by the range of the mean percent differences from the nominal concentrations over all concentrations.

Using two-way analysis of variance with replication, the analytical method was tested for the influences of drug concentration, freezing, and the interactive term (the product of concentration and freezing) on the assayed concentrations. Using the null hypothesis, a value of less than 0.05 was considered significant.

Estimates of the pharmacokinetic parameters were determined from plasma data using the SAS program NLIN [3].

Animal studies

Three male Beagle dogs received an intravenous dose equivalent to 5 mg of fezolamine (as fumarate) per kg body weight. Blood samples of 3 ml were taken at appropriate intervals, using oxalate-fluoride anticoagulant, and the plasma was separated by centrifugation. Plasma samples were frozen in a laboratory freezer until analysis.

Urine samples were collected using an indwelling catheter for the first two hours and from individual metabolic cages thereafter. Samples were frozen until analyzed.

RESULTS AND DISCUSSION

Typical chromatograms generated from the computer raw data files are shown in Fig. 2 with extracted plasma and urine samples spiked with only the internal standard shown in A and B, respectively. The chromatograms in Fig. 2C and D show extracted samples which also contain the didesmethyl metabolite (Fig. 1, III), the desmethyl metabolite, and the parent drug. The chromatograms in Fig. 2E and F, respectively, were obtained from plasma and urine samples taken from beagle dogs receiving an intravenous bolus of fezolamine fumarate at 5 mg (base) per kg. Equivalent peaks were identified from rat urine as the desmethyl metabolite and fezolamine by chromatographic isolation and mass spectral analysis. A very small peak observed at about 10 min in Fig. 2E was initially thought to be the didesmethyl metabolite; however, mass spectral analysis indicates that at least two as yet unidentified metabolites are present [4]. Two small peaks eluting between 6 and 8 min in dog urine (Fig. 2F) were not observed in pre-medication samples and are presumed metabolites. Slightly longer separation times were needed to achieve baseline separation of these two peaks from the internal standard.

Linearity was observed for fezolamine and its desmethyl metabolite in both plasma and urine with r^2 values invariably greater than 0.99 for standard curves between 0.0 and 500 ng/ml. We have also observed linearity of response with standard curves extended to 2000 ng/ml. The mean slopes for
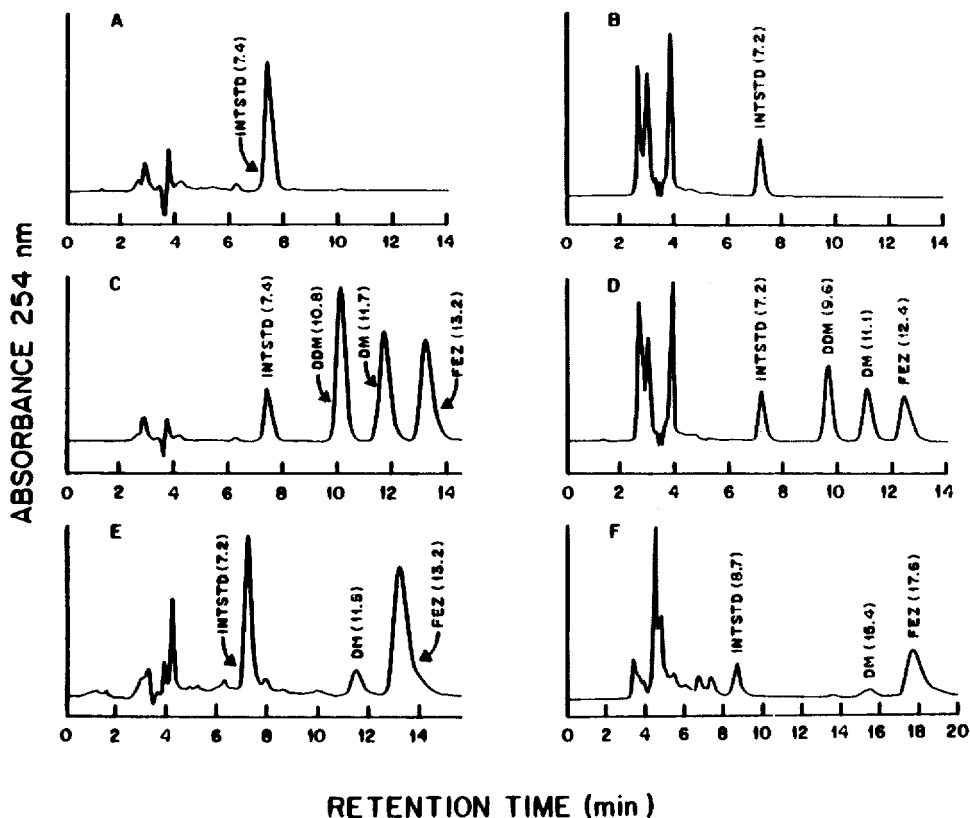
Fig. 2. Chromatograms of fezolamine from HPLC analyses. (A) Human plasma spiked with 5 μg of the internal standard; (B) human urine spiked with 5 μg of the internal standard; (C) human plasma spiked to 500 ng/ml with fezolamine, its desmethyl and didesmethyl metabolites; (D) human urine spiked to 500 ng/ml with fezolamine, its desmethyl and didesmethyl metabolites; (E) dog plasma taken 3 h after intravenous injection of 5 mg (base) fezolamine per kg; (F) dog urine collected between 8 and 24 h after intravenous injection of 5 mg (base) fezolamine per kg. Peaks: INTSTD = internal standard (Bupivacaine); DM = desmethyl metabolite; DDM = didesmethyl metabolite; FEZ = fezolamine.

fezolamine and desmethyl fezolamine in plasma were $3.93 \cdot 10^{-3}$ and $4.44 \cdot 10^{-3}$, respectively, and in urine $1.85 \cdot 10^{-3}$ and $2.20 \cdot 10^{-3}$, respectively. Intercepts tended to be slightly less than zero, but were not significantly different from zero. The minimum quantifiable levels determined from these regression lines were all less than 10 ng/ml and approach the limits of detection (approximately 5 ng/ml).

The results of the single-blind validation for the assays are shown in Table I for fezolamine and in Table II for the desmethyl metabolite. In the plasma analysis, the presence of drug or metabolite at less than 10 ng/ml was observed in a few of the samples containing no drug or metabolite (blank), indicating the unreliability of the assay near the limit of detection. A summary of the analytical parameters is presented in Table III. The precision and accuracy of both assays are within acceptable limits for the intended use and within accepted limits for similar compounds [5].

Two aspects of drug and metabolite recovery became apparent during the

TABLE I

SUMMARY OF DATA FROM ANALYSIS OF FEZOLAMINE IN PREPARED SAMPLES

Urine			Plasma		
Nominal concentration (ng/ml)	Concentration found (ng/ml)		Nominal concentration (ng/ml)	Concentration found (ng/ml)	
	Fresh[*]	Frozen[**]		Fresh[***]	Frozen[§]
0	< MQL	< MQL	0	< MQL	< MQL
	< MQL	< MQL		< MQL	< MQL
	< MQL	< MQL		< MQL	8.5
67.5	67.8	65.8	60	53.4	66.0
	64.6	64.8		60.2	61.8
	65.9	65.8		58.1	58.9
Mean (ng/ml)	66.1	65.5		57.2	62.2
S.E.M.[§§] (%)	1.4	0.51		3.5	3.3
Mean difference (%)	−2.1	−3.0		−4.6	3.7
80	80.1	78.9	150	152	139
	79.3	87.9		152	151
	73.4	83.1		140	145
Mean (ng/ml)	77.6	83.3		148	145
S.E.M. (%)	2.7	3.1		2.6	2.3
Mean difference (%)	−3.0	4.1		−1.2	−3.4
187.5	192	196	250	250	227
	185	185		270	243
	186	182		253	237
Mean (ng/ml)	188	188		257	236
S.E.M. (%)	1.2	2.3		2.5	1.9
Mean difference (%)	0.09	0.09		3.0	−5.7
400	399	397	375	382	386
	397	388		378	383
	413	391		390	382
Mean (ng/ml)	403	392		384	384
S.E.M. (%)	1.2	0.67		0.9	0.3
Mean difference (%)	0.75	−2.0		2.3	2.3
			475	475	459
				470	468
				−§§§	470
Mean (ng/ml)				473	466
S.E.M. (%)				0.5	0.7
Mean difference (%)				−0.55	−2.0

[*] Analyzed immediately after preparation, minimum quantifiable level (MQL) = 2.5 ng/ml.
[**] Frozen for four days before analysis, MQL = 4.2 ng/ml.
[***] Analyzed immediately, MQL = 6.2 ng/ml.
[§] Frozen for five days, MQL = 6.0 ng/ml.
[§§] S.E.M. = Standard error of the mean.
[§§§] Sample lost.

development of a suitable extraction procedure. The recoveries of both the parent drug and the desmethyl metabolite were greatly increased by the addition of 4 ml of water prior to denaturation of the plasma proteins. This phenomenon is probably associated with a change in the binding of the com-

TABLE II

SUMMARY OF DATA FROM ANALYSIS OF MONODESMETHYL METABOLITE IN PREPARED SAMPLES

Urine			Plasma		
Nominal concentration (ng/ml)	Concentration found (ng/ml)		Nominal concentration (ng/ml)	Concentration found (ng/ml)	
	Fresh*	Frozen**		Fresh***	Frozen §
0	< MQL	< MQL	0	< MQL	< MQL
	< MQL	< MQL		9.0	< MQL
	< MQL	< MQL		9.4	< MQL
67.5	67.4	65.4	60	54.9	61.7
	64.6	66.5		53.7	60.5
	67.3	67.4		— § §	56.3
Mean (ng/ml)	66.4	66.4		54.3	59.5
S.E.M. § § § (%)	1.4	0.86		1.0	2.8
Mean difference (%)	−1.6	−1.6		−9.5	−0.9
80	80	79.3	150	143	138
	79.9	83.7		147	151
	74.3	81.2		144	139
Mean (ng/ml)	78.1	81.4		145	143
S.E.M. (%)	2.4	1.6		0.8	3.0
Mean difference (%)	−2.4	1.8		−3.6	−4.7
187.5	193	193	250	231	224
	187	185		268	242
	187	185		248	242
Mean (ng/ml)	189	188		249	236
S.E.M. (%)	1.1	1.4		4.3	2.6
Mean difference (%)	0.80	0.09		−0.3	−5.7
400	402	395	375	373	381
	400	388		374	380
	406	393		394	376
Mean (ng/ml)	403	392		380	379
S.E.M. (%)	0.44	0.52		1.9	0.4
Mean difference (%)	0.67	−2.0		1.4	1.0
			475	471	467
				473	467
				— § §	470
Mean (ng/ml)				472	470
S.E.M. (%)				0.3	0.6
Mean difference (%)				−0.7	−1.0

*Analyzed immediately after preparation, minimum quantifiable level (MQL) = 5.1 ng/ml.
**Frozen for four days before analysis, MQL = 3.3 ng/ml.
***Analyzed immediately, MQL = 7.5 ng/ml.
§ Frozen for five days, MQL = 9.4 ng/ml.
§ § Sample lost.
§ § § S.E.M. = Standard error of the mean.

TABLE III

SUMMARY OF STATISTICAL ANALYSES

Compound	Recovery (%)	Accuracy (%)	Precision (%)	MQL* (ng/ml)	S.E.M.** (%)
Plasma					
Fezolamine (I)	103	−5.7 to 3.7	± 3.8	6.1	0.1
Desmethyl metabolite (II)	89.4	−9.5 to 2.2	± 3.8	8.4	1.0
Urine					
Fezolamine (I)	93.7	−3.0 to 4.1	± 3.3	3.4	0.85
Desmethyl metabolite (II)	91.9	−2.4 to 1.8	± 2.3	4.2	0.92

*MQL = Minimum quantifiable level.
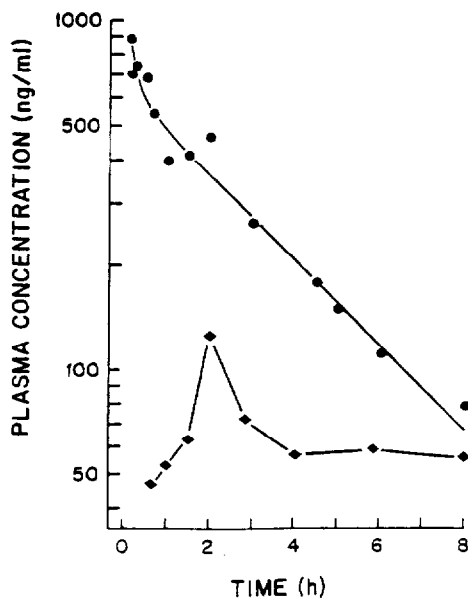**S.E.M. = Standard error of the mean MQL.

Fig. 3. Mean plasma concentrations of fezolamine (•) and its desmethyl metabolite (♦) for three beagle dogs receiving 5 mg (base) fezolamine per kg as an intravenous bolus.

pounds to plasma proteins. The second aspect was the use of silanized glassware. While recovery of the parent drug was unaffected by silanization, the recovery of the metabolite was greatly enhanced. Similar problems with drug binding have been reported for tricyclic antidepressants [5].

The percent differences between nominal values and the assayed values (Tables I and II) were submitted to analysis of variance to determine whether there were any systematic effects due to concentration, freezing and the interactive term, the product of concentration and freezing. With one exception, these influences were all insignificant at $\alpha = 0.05$. This exception was the influence of the interactive term on the fezolamine plasma analysis at $\alpha = 0.02$. While the interactive is statistically significant, the total range of mean assayed values for both fresh and frozen samples at all concentrations is −5.7% low to +3.7% high. The effect is therefore trivial in routine analysis.

The assay methods were used to follow the time course of the disappearance of the drug and metabolite in three beagle hounds following intravenous injection of 5 mg/kg fezolamine. To obviate species differences in the methods,

prepared samples of known concentrations in dog plasma and urine were included in the analyses. The assayed concentrations of both parent drug and desmethyl metabolite were within a few percent of the nominal value. The mean plasma levels of the drug (circles) and the desmethyl metabolite (diamonds) are shown in Fig. 3 as the logarithm of the plasma concentration plotted as a function of time. The elimination of the drug can be described by two first-order processes, a rapid distribution phase and a slower elimination phase. The mathematical equation [6] was solved using non-linear regression, and the pharmacokinetic parameters are shown in Table IV. The mean distribution rate constant is 4.1 h^{-1} and corresponds to a mean half-life of 11 min; the mean disposition rate constant is 0.28 h^{-1} with a corresponding half-life of 2.6 h. Peak plasma concentration of the metabolite was observed 2 h after medication. As can be seen from the figure, the elimination of metabolite takes place very slowly after 4 h.

The amounts of fezolamine and its desmethyl metabolite recovered from urine collected at various intervals are shown in Table V. The amount of drug recovered over any given interval varied considerably between animals as did the amount of metabolite. The percentage of the parent drug recovered was less than 5% in all of the animals and the percentage of the metabolite recovered was less than 1% for the 48-h experimental period.

In summary, accurate, sensitive and reproducible HPLC assays for the quantitation of fezolamine and its desmethyl metabolite in plasma and urine have been developed. These methods have been used to determine pharmacokinetic parameters in beagle hounds.

TABLE IV

PHARMACOKINETIC PARAMETERS OF FEZOLAMINE IN BEAGLE DOGS RECEIVING 5 mg/kg INTRAVENOUS INFUSION BOLUS

Parameter	Dog A	Dog B	Dog C	Mean ± S.E.M.[*]		
Weight (kg)	13.2	13.8	10.8	12.6	±	0.9
Dose administered as free base	66.0	69.0	54.0	63.0	±	4.6
α[**] (h^{-1})	3.0	6.3	3.0	4.1	±	1.1
β[**] (h^{-1})	0.24	0.25	0.34	0.28	±	0.03
A[**] (ng/ml)	387	620	384	464		± 78
B[**] (ng/ml)	529	629	589	582		± 29
k_{12}[***] (h^{-1})	1.0	2.8	0.9	1.6	±	0.6
k_{21}[***] (h^{-1})	1.8	3.3	2.0	2.4	±	0.5
k_{10}[***] (h^{-1})	0.39	0.49	0.52	0.47	±	0.04
Disposition half-life (h)	2.9	2.8	2.0	2.6	±	0.3
Central compartment volume of distribution V_c (l/kg)	5.5	4.0	5.1	4.9	±	0.4

[*]S.E.M. = Standard error of the mean.
[**]Plasma concentration = $Ae^{-\alpha t} + Be^{-\beta t}$ where t is time after dosing.
[***]Derived from the model: Central compartment $\overset{k_{12}}{\underset{k_{21}}{\rightleftarrows}}$ Tissue compartment

$\downarrow k_{10}$

Urine

TABLE V

URINARY EXCRETION OF FEZOLAMINE AND ITS DESMETHYL METABOLITE FROM BEAGLE DOGS RECEIVING 5 mg/kg FEZOLAMINE INTRAVENOUSLY

Drug was administered as intravenous bolus at 5 mg/kg. Urine was collected by catheterization for 2 h and from metabolic cages thereafter.

Time after	Fezolamine (I) (μg)			Desmethyl metabolite (μg)		
	Dog C (10.8 kg)	Dog A (13.2 kg)	Dog B (13.8 kg)	Dog C (10.8 kg)	Dog A (13.2 kg)	Dog B (13.8 kg)
0—1	22.1	—*	26.0	<MQL**	—*	3.7
1—2	230	55.0	66.9	11.2	4.3	7.3
2—3	12.8	96.0	—*	1.3	8.4	—*
3—5	53.7	167	104	10.5	15.4	24.9
5—6	—*	—*	75.6	—*	—*	22.2
6—8	64.0	7.6	19.5	7.4	0.50	7.7
8—24	2120	648	184	316	46.2	47.7
24—30	10.6	44.8	12.7	5.4	4.3	8.4
30—48	51.2	49.0	18.6	<MQL**	9.0	14.3
Total (μg)	2560	1070	507	352	88.1	136
Percentage of dose	4.7	1.6	0.7	0.6***	0.1***	0.2***

*No sample obtained at this time point.
**Less than minimum quantifiable level (MQL).
***Expressed as equivalents to the parent drug.

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